Characterization of levels and cellular transfer of circulating lipoprotein-bound microRNAs

Wagner, Jasmin ; Riwanto, Meliana ; Besler, Christian ; Knau, Andrea ; Fichtlscherer, Stephan ; Röxe, Tino ; Zeiher, Andreas M ; Landmesser, Ulf ; Dimmeler, Stefanie

Abstract: OBJECTIVE: MicroRNAs are important intracellular regulators of gene expression, but also circulate in the blood being protected by extracellular vesicles, proteins, or high-density lipoprotein (HDL). Here, we evaluate the regulation and potential function of HDL- and low-density lipoprotein-bound miRs isolated from healthy subjects and patients with coronary artery disease. APPROACH AND RESULTS: HDL-bound miRs with known effects in the cardiovascular system were analyzed in HDL isolated from healthy subjects (n=10), patients with stable coronary artery disease (n=10), and patients with an acute coronary syndrome (n=10). In HDL from healthy subjects, miR-223 was detected at concentrations >10 000 copies/µg HDL, and miR-126 and miR-92a at about 3000 copies/µg HDL. Concentrations of most miRs were substantially higher in HDL as compared with low-density lipoprotein. However, HDL-bound miR-223 contributed to only 8% of the total circulating miRs. The signatures of miRs varied only slightly in HDL derived from patients with coronary artery disease. We did not observe a significant uptake of HDL-bound miRs into endothelial cells, smooth muscle cells, or peripheral blood mononuclear cells. However, patient-derived HDL transiently reduced miR expression particularly when incubated with smooth muscle and peripheral blood mononuclear cells. CONCLUSIONS: Circulating miRs are detected in HDL and to a lesser extent in low-density lipoprotein, and the miR-signatures are only slightly altered in patients with coronary artery disease. Lipoprotein-bound miRs were not efficiently delivered to endothelial, smooth muscle, and peripheral blood mononuclear cells suggesting that the lipoprotein-associated pool of miRs is not regulating the function of the studied cells in vitro.

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Characterization and function of HDL-bound microRNAs in cardiovascular disease

Jasmin Wagner[1], Meliana Riwanto[2], Christian Besler[2], Andrea Knau[1], Stephan Fichtlscherer[3], Tino Röxe[1], Andreas Zeiher[3], Ulf Landmesser[2], Stefanie Dimmeler[1]

[1]Institute of Cardiovascular Regeneration, Centre for Molecular Medicine, Goethe-University Frankfurt, Frankfurt, Germany

[2] Cardiovascular Research, Institute of Physiology, University of Zurich, Zurich, Switzerland

Cardiology, Cardiovascular Center, University Hospital Zurich, Zurich, Switzerland

[3] Division of Cardiology, Department of Medicine III, Center of Molecular Medicine, Goethe University Frankfurt, Frankfurt, Germany

Address for correspondence [#]:

Stefanie Dimmeler, PhD

Institute of Cardiovascular Regeneration, Centre for Molecular Medicine

University of Frankfurt, Theodor-Stern-Kai 7, 60590 Frankfurt, Germany
Fax: +49-69-6301-7113, Phone: +49-69-6301-7440

e-mail: dimmeler@em.uni-frankfurt.de

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Objective
MicroRNAs are important intracellular regulators of gene expression, but also circulate in the blood being protected by extracellular vesicles, proteins or high density lipoprotein (HDL). Here we evaluate the regulation and function of HDL-bound miRs in cardiovascular disease.

Methods and Results
HDL-bound miRs with known effects in the cardiovascular system were analyzed in HDL isolated from healthy subjects (HS; n=10), patients with stable coronary artery disease (n=10) and patients with an acute coronary syndrome (n=10) by quantitative real-time PCR. In HDL from HS, miR-223 was detected at concentrations >10,000 copies/µg HDL, and miR-126 and miR-92a at about 3000 copies/µg HDL. miR concentrations were higher in HDL compared to LDL. The signatures of miRs varied only slightly in HDL derived from patients with coronary disease, which showed a reduction of HDL-bound miR-92a.
We did not observe a significant uptake of HDL-bound miRs into endothelial cells, smooth muscle cells or peripheral blood mononuclear cells. However, patient-derived HDL transiently reduced miR expression particularly when incubated with smooth muscle and peripheral blood mononuclear cells.

Conclusion
Circulating miRs are detected in HDL and to a less part also by LDL. However, HDL-bound miRs are not efficiently taken up by endothelial cells, smooth muscle cells and peripheral blood mononuclear cells, in contrast miRs are transiently reduced in these cells.
Certainly, the present study does not exclude an uptake of HDL-bound miRs in vivo for instance in atherosclerotic lesions or foam cells.

Key words: microRNA, high density lipoprotein, circulation, cardiovascular disease, endothelial cells

Introduction

MicroRNAs (miRs) are endogenously expressed small non-coding RNAs that regulate gene expression on the posttranscriptional level by degradation or translational repression of target mRNAs. Primary microRNA transcripts (pri-miRs) are processed by the endonucleases Drosha and Dicer into precursor miRs (pre-miRs) and mature miRs. Incorporated into the RNA-induced silencing complex, miRs bind to hundreds of target mRNAs and thereby control gene expression patterns. MiRs exhibit important functions in the cardiovascular system[1-3]. For example, the endothelial enriched miR-126 controls vascular integrity[4, 5] and provides atheroprotective effects[6]. In contrast, miR-92 blocks blood vessel growth and impairs endothelial cell functions[7]. The miR-143/145 cluster is highly expressed in vascular smooth muscle cells and prevents atherosclerotic lesion formation by influencing vascular smooth muscle cell phenotypes[8-10]. Several inflammation-associated miRs have been described such as miR-146 and miR-155, which might influence the pro-inflammatory environment and thereby may affect atherosclerotic lesion formation[11]. MiR-targeted therapeutics might become a highly interesting novel approach to treat cardiovascular disease[12].

Although miRs act intracellular, they can be released and detected in circulating blood and might be used as disease biomarkers[13]. MiRs are remarkably stable in plasma indicating that they are protected from RNase-dependent degradation. Several studies showed that miRs are protected by lipid vesicles or protein conjugates[14-16]. MiRs are actively secreted in microvesicles[17, 18], contained in apoptotic bodies[6] or bound to proteins like Argonaute2[16]. Interestingly, endothelial cell-derived apoptotic bodies were shown to contain high levels of miR-126 and reduce atherosclerosis when injected in mice models[6]. Likewise, microvesicles derived from flow-exposed endothelial cells are enriched in atheroprotective miRs and inhibit atherosclerotic lesion formation in ApoE-/- mice[19]. Recent studies additionally suggest that circulating high density lipoprotein (HDL) can bind and transport endogenous miRs, particularly miR-223, and delivers those to hepatocytes[14]. The functional properties of miRs that are bound to HDL in the cardiovascular system, however, are unclear.

Elevated high density lipoprotein (HDL) serum levels are associated with reduced risk for coronary artery disease (CAD) and HDL from healthy subjects was shown to have potential atheroprotective effects by several mechanisms. However, HDL-raising using the CETP inhibitors torcetrapib or dalcetrapib has failed to reduce cardiovascular events in patients with coronary disease so far. That may in part be related to different vascular effects of HDL from patients with CAD as compared to HDL from healthy subjects[20].

In the present study, we addressed whether circulating HDL and also low density lipoprotein (LDL) might contain miRs implicated in cardiovascular disease and may control vascular functions by delivering miRs. Second, we analyzed HDL-bound miRs in patients with stable CAD and acute coronary syndrome (ACS) as compared to HDL from healthy subjects to determine whether the miRs signature of HDL is different in patients with coronary disease. Due to the limited amount of RNA we could derive from plasma HDL we focused our analysis on a subset of vascular and inflammation-associated miRs. More precisely, miR-92a, miR-126, miR-150 and miR-378, which are highly expressed in endothelial cells, and regulate angiogenesis [5, 7, 21], the smooth muscle specific miR-145, which is the most abundant miR in the vascular wall [22], and miR-30c that is highly expressed in cardiac myocytes [23] but also is enriched in endothelial cells. miR-146a and miR-155 are involved in the control of inflammation associated processes [24, 25] and miR-223 is liver-specific and was shown to be bound to HDL in a previous study [14]. Among the miRs tested particularly miR-145 and miR-150 were shown to be released from cells in microvesicles [19, 26].

Here we demonstrate that miRs known to be relevant in the cardiovascular system are bound to HDL albeit at low copy numbers. In contrast even less miRs are bound to LDL. Transfer of artificially bound *Caenorhabditis elegans* miRs by HDL to endothelial cells was very inefficient. However, addition of HDL from healthy subjects to endothelial, smooth muscle or peripheral blood mononuclear cells reduced endogenous miR levels. The miR signature in HDL isolated from patients with coronary disease was only slightly changed as compared to miRs in HDL derived from healthy subjects. However, patient-derived HDL had a profoundly changed biological effect on miR expression in

peripheral blood mononuclear cells. Thus, HDL induced down-regulation of miR levels is even increased after short time points whereas HDL treatment for longer time point promoted up-regulation of miR levels.

Material and Methods

Patient Population

Patients with stable coronary artery disease (CAD) or an ACS (STEMI or NSTEMI) and healthy subjects (without cardiovascular risk factors) were recruited at the University Hospital of Zurich. The diagnosis of stable CAD or an ACS was made according to the guidelines of the American College of Cardiology/American Heart Association task force[27, 28]. Patients with an ACS (STEMI and NSTEMI) were recruited if they presented within 12 hours after the onset of symptoms and were in a fasting state for at least 12 hours. Further exclusion criteria were accompanying infectious, inflammatory or autoimmune disorders, advanced kidney or liver failure, diabetes, neoplastic disorders and a history of major surgery or trauma within the previous month.

HDL and LDL isolation

HDL and LDL were isolated from plasma of patients with stable CAD or ACS, and healthy subjects by sequential ultracentrifugation (*d* = 1.063-1.21 g/ml) using solid potassium bromide (Merck KGaA, Germany) as described in detail previously [29-31].

Cell culture

Human umbilical vein endothelial cells (HUVEC) were purchased from Lonza and cultured in endothelial basal medium (EBM; Lonza) supplemented with EGM-SingleQuots (Lonza), and 10% fetal calf serum (FCS; Invitrogen) until the third passage as previously described[32].
Human Vascular Smooth Muscle Cells (SMC) were purchased from Lonza and cultured in SmBM basal medium (Lonza) supplemented with 5% FBS and SmGM-2 SingleQuots (Lonza). SMC were passaged after reaching 80% confluency and used until the third passage.
Peripheral blood mononuclear cells (PBMC) were isolated by density gradient centrifugation with Biocoll (Biochrom) from buffy coat preparations of healthy human volunteers. Cells were transferred in RPMI-1640 medium (Sigma) and directly seeded for incubation with HDL.

HDL- and LDL-miR cell culture delivery experiments

To analyze the uptake of endogenously HDL- and LDL-bound miRs by HUVEC, SMC and PMNC, cells were seeded in 24well plates (150,000 HUVEC or SMC/well and 3,000,000 MNC/well). After attachment, HUVEC or SMC were starved in EBM or SMC medium with supplements and 0.5% FCS for 10 hours. PMNC were directly incubated with HDL without starving in RPMI medium. 50 µg/ml or 1 mg/ml purified HDL was added to the medium, and RNA was isolated after 1/6/16/24 hours incubation at 37°C.
To investigate the uptake of artificially HDL-bound miRs by HUVEC, a complex of purified HDL and *Caenorhabditis elegans* microRNA-39 (cel-miR-39) was formed. Therefore, 80 µg purified HDL was incubated with 2 µM mature cel-miR-39 (RNA oligonucleotide, Sigma-Aldrich), cel-miR-39 mimic (Life Technologies) or cel-miR-39 precursor (Ambion) for 1 hour at 37°C and overnight at 4°C, rotating. Unbound miRs were removed by dialysis. The dialysate was applied to HUVEC (100,000 cells/well, 24well plate), and incubated for 24 hours at 37°C before RNA isolation.

RNA Isolation and Quantitative Real-time PCR

RNA was isolated from 10 µl purified HDL. 10 µl HDL results from approximately 250 µl blood serum corresponding to HDL protein concentration of 18-25 µg/µl and resulting in 0.72 – 1 µg HDL/µl serum. Furthermore, RNA was isolated from 50 µl purified LDL with LDL protein concentration of 3-7 µg/µl. Isolation was carried out with the blood derivate–specific TRIzol BD from Sigma as described previously[13]. For RNA isolation from Plasma TRIzol BD was used in combination with the miRNeasy Kit. RNA from cells was isolated using the miRNeasy Kit according to the manufactures protocol (Qiagen). For normalization of miR expression the sample in TRIzol was supplemented with 5 nmol/l *C. elegans* miR-39 (RNA oligonucleotide, Sigma-Aldrich).

1 µl RNA of 20 µl total RNA isolated from 10 µl purified HDL or 50 µl purified LDL respectively or 1 µl of 30 µl total RNA from 250 µl EDTA-plasma was reverse transcribed using TaqMan reverse Transcription Kit (Life Technologies) according to the manufactures instructions. For detection of miR expression in cells, 10 ng total RNA were reverse transcribed as described above. 3 µl of the product was used for detection of miRs by quantitative PCR using Taqman microRNA Assays (Life Technologies) and Applied Biosystems StepOnePlus machine. RNU6 was used for normalization. For quantitative detection of pri- and pre-miRs and mRNA (Dicer, Drosha) 600 ng RNA were reverse transcribed in a 40 µl reaction using MuLVRT (Life Technologies). SYBR-green qRT-PCR was performed using 6 µl 1:5 diluted cDNA. Human ribosomal P0 was used for normalization. For calculation of miR copy numbers a standard curve run for each miR with known picomolar concentrations of recombinant miRs (RNA oligonucleotides, Sigma-Aldrich) was performed in parallel. To calculate the copy number per cell we assumed a RNA concentration of 20 pg per cell.

Statistical analysis

Data were analyzed with Graphpad Prism 5 using unpaired student's t-tests with Welch´s correction when comparing two conditions, or one-way ANOVA with Bonferroni correction for multiple comparisons. Correlation was analyzed using two-sided Spearman´s method. A significance level of $p<0.05$ was considered significant. Data are presented as mean with error bars depicting the standard error of the mean (SEM).

Results

Quantification of human HDL and LDL containing microRNAs

First we determined, whether vascular and inflammation-associated miRNAs are bound to HDL and LDL at biological relevant concentrations. HDL and LDL were isolated from healthy subjects (n=10 for HDL; n=5 for LDL) and miRs were detected by quantitative real-time PCR using recombinant miRs for quantification.

Consistent with previous findings, miR-223 was highly bound to HDL at concentrations of >10,000 copies/µg HDL (Fig. 1A). The endothelial-enriched miR-126 was detected in HDL with about 2,800 copies/µg HDL. Likewise, miR-92a, which is highly expressed in endothelial cells, was detected in HDL at a similar concentration (Fig. 1A). miR-150, which is released by monocytes and controls endothelial cell migration[26], was detected albeit at a lower level.The smooth muscle enriched miR-145, inflammation associated miRs such as miR-146a and miR-155 and the metabolically controlled miR-378 were detected although at levels below 120 copies/µg HDL (Fig. 1A).

Next we determined the miR concentrations bound to LDL. Similar to HDL, miR-223 is also highest abundant in LDL. However, only 1,500 copies/µg LDL compared to <10,000 copies/µg HDL miR-223 were detected (Fig. 1B). The concentrations of LDL-bound miR-126, miR-145, miR-150, miR-378 and miR-30c were extremely low (<10 copies /µg LDL). In contrast the pro-atherosclerotic miR-155 is higher abundant in LDL compared to HDL (Fig 1B).

Further on, we examined to what extend HDL bound miRs contribute to the total pool of circulating miRs. Therefore, we measured the miR concentration in the total plasma from the same individuals of whom HDL was isolated (Fig. 1C) and calculated the percentage of HDL-associated miRs compared to the total plasma concentrations. HDL bound miR-223 contributes to about 8% of the total plasma pool (Fig. 1D). About 4-5% of circulating miR-126 and miR-378 were associated to HDL, whereas 1-2% miR-92a and miR-150 and below 1% of the other miRs were HDL bound (Fig. 1D). Overall a highly significant correlation between the absolute concentration of miRs that are bound to HDL and the detected circulating miRs in plasma was found (Fig. 1E). However, some miRs (e.g. miR-223, miR-378, miR-146a) varied only in the plasma fraction independently on the concentration in HDL and others (miR-155, miR-92a) differ in HDL and are more constant in plasma (Fig. 1E). In patients also a significant correlation of HDL-bound miRs and plasma miRs were observed (Suppl. Figure 1). However, consistent with the data obtained in healthy controls, miR-223 and miR-378 are relatively constantly expressed in HDL but differ in plasma.

HDL- and LDL-miR signatures in patients with coronary disease

HDL derived from patients with coronary disease shows a significantly impaired vasculoprotective function[31]. Therefore, we examined whether miR signatures might be different in HDL isolated from these patients. HDL was isolated from patients with stable CAD (n=10) and patients with an ACS (n=10) (for patient characteristics see supplemental table 1). miR levels in HDL isolated from patients with stable CAD did not significantly differ from those measured in HDL derived from healthy subjects. In contrast, in patients with ACS, the absolute copy numbers of HDL-bound miR-92, miR-146, and miR-30c were significantly reduced as compared to the miR concentrations measured in HDL derived from healthy subjects (Fig. 2A).

In LDL, no significant differences in miR levels isolated from healthy subjects and patients with CAD and ACS could be detected (Suppl. Fig. 2). Only miR-126 is slightly elevated in LDL of patients with ACS.

To determine whether there is a change in the distribution of circulating miRs in patients with coronary disease, we additionally measured the miR concentrations in plasma of the same patients and calculated the percentage of HDL-bound miRs in total plasma. We observed that the percentage of HDL-bound miR-92a was reduced in patients with CAD and ACS. Moreover, the level of HDL-bound miR-155 was significantly increased in patients with ACS but generally was very low (< 0.5 %) (Fig. 2B). All other analyzed HDL-bound miRs showed no significant differences between healthy controls and patients with CAD.

Transfer of HDL –bound miRs to cultured endothelial cells

Previous studies suggested that HDL bound miRs can be transferred to SRB1 overexpressing baby hamster kidney cells and hepatocytes[14]. Therefore, we examined whether HDL might also deliver miRs to endothelial cells.

Since HDL might influence the biosynthesis and processing of endogenously expressed miRs in endothelial cells, we incorporated an artificial *C. elegans* miR (cel-miR-39) in HDL in vitro. After removal of unbound miRs, we determined the uptake in human umbilical venous endothelial cells (HUVEC) (Fig. 3A). We analyzed the uptake of the mature single-stranded cel-miR-39 as well as two different double-stranded precursor miRs, cel-miR-39 mimic and cel-miR-39 pre-miR (cel-pre-miR-39). Only around 5 copies HDL-bound mature cel-miR-39 and cel-miR-39 mimic were taken up per cell, whereas free mature miRs, which were not bound to HDL did not enter the cells (Fig. 3B). HDL-bound cel-miR-39 precursor is taken up by endothelial cells in similar amounts, but in contrast much higher levels (approximately 50 copies per cell) of HDL-free cel-pre-miR-39 are incorporated in the cells (Fig. 3B).

Together these data demonstrate that HDL bound synthetic miRs can be transferred to cultured endothelial cells, however, only very low copy numbers were detected in the recipient cells.

Effect of HDL on miR expression in different cell types

Because of the low copy numbers of cel-miR-39 detected in HUVEC in the transfer experiments (Fig. 3A/B), we further analyzed, whether endogenous HDL-bound miRs can be delivered to endothelial (HUVEC), smooth muscle (SMC) and peripheral blood mononuclear cells (PBMC). We incubated HUVEC, SMC and PBMC with HDL and measured the expression of those miRs, which were associated to HDL at concentrations >2000 copies/µg HDL namely miR-223, miR-92a and miR-126 (Fig 1A). No significant miR expression changes could be observed in HUVEC after incubation with HDL for different time points (Fig. 3D). However, all three analyzed miRs showed a slightly decreased expression in HUVEC after 1 hour incubation with HDL (Fig. 3D). Moreover, miR-223 which is highly bound to HDL and very low expressed in cultured endothelial cells (CT=30), but induces angiogenesis if overexpressed in cultured endothelial cells (Suppl. Fig. 3), was decreased by HDL treatment for 16 hours (Fig. 3D). Furthermore, increasing the HDL concentration to 1 mg/ml and extending the incubation time to 24 hours did not increase the levels of miR-126, miR-92a and miR-223 in endothelial cells (Suppl. Fig. 4).

In SMCs, miR-92a and miR-126 were slightly reduced after 1 hour and not changed after 6 hours incubation with HDL (Fig. 3E). miR-223 is only very low expressed in SMC (CT=28) and expression is not significantly changed after HDL treatment (Fig. 3E). Most profound changes were observed in HDL-treated PBMC. HDL incubation reduced the levels of miR-126 and miR-223 by about 15%. miR-92a is only slightly reduced after 1 hour, but also significantly reduced after 6 hours incubation with HDL (Fig. 3F).

In summary, no transfer of natively HDL bound miRs to endothelial, smooth muscle and mononuclear cells could be documented. In contrast, HDL from healthy subjects overall tend to reduce the levels of the measured miRs in all analyzed cell types.

Effects of HDL from patients with coronary disease on different cell types

Previous studies demonstrated that the HDL-miR profile is significantly different in patients with familial hypercholesterolemia compared to healthy subjects[14]. However, the impact of coronary disease is unknown. Therefore, we studied the effect of HDL derived from patients with coronary disease on miR expression in different cell types. Likewise to HDL from healthy subjects, HDL from patients has no significant effect on miR expression in endothelial cells (Fig. 4A). However, the slight reduction of miR expression after 1 hour incubation with HDL from healthy subjects is abrogated when using patient derived HDL (Fig. 4A). In contrast to the marginal reduction of miR-92a and miR-126 in SMC after treatment with HDL from healthy subjects for 1 hour, HDL from patients with CAD or ACS increased the levels of miR-92a and miR-126 in SMC (Fig. 4B). No effect on miR expression

was observed in SMC after longer incubation with HDL from healthy subjects or patients with coronary disease (Fig. 4B). In PBMCs, miR expression is profoundly decreased after incubation with HDL from patients with coronary disease. All three miRs are reduced by more than 60% after incubation with HDL from patients with CAD, and more than 50% after treatment with HDL from patients with ACS (compared to about 15% reduction with HDL from healthy subjects) for 1 hour (Fig. 4C). On the contrary 6 hours after incubation of PBMC with HDL from patients with coronary disease all analyzed miRs are significantly increased again compared to treatment with HDL from healthy subjects (Fig. 4C).

Influence of HDL on miR biogenesis in peripheral blood mononuclear cells

Because we obtained the most profound effect on miR expression change when incubating PBMC with HDL, we further tried to elucidate the mechanism how HDL influences miR levels. Therefore we estimated expression levels of primary (pri-) and precursor (pre-) miRs (Fig. 5A) and mRNA levels of Drosha and Dicer, the enzymes responsible for miR maturation (Fig. 5B) after treatment with HDL from healthy subjects as well as HDL from patients with coronary disease.

PBMC treatment with HDL has no effect on pri- and pre- miR-92a and miR-223. Only pri-miR-223 expression is slightly but significantly reduced (14+-3%) after incubation with HDL from patients with ACS for 6 hours (Fig. 5A). In contrast pri-, pre- and mature miR-126 levels are comparably affected by HDL treatment (Fig. 5A and Fig. 4C). More precise, pri- and pri-126 expression is significantly reduced after 1 hour incubation with HDL from healthy subjects and patients and pri- and pre-miR-126 levels are increased after 6 hour incubation with patient derived HDL, whereas HDL from healthy subjects does not lead to a change in pri- and pre-miR-126 expression after 6 hours treatment (Fig. 5A).

Besides a slight but significant up regulation of Drosha after 1 hour incubation with HDL from patients with CAD (11+-2%), both Drosha and Dicer expression are not changed by HDL treatment (Fig. 5B).

In summary, HDL seems to affect miR-126 transcription whereas the modulation of miR-92a and miR-223 levels is likely due to effects on miR processing.

Discussion

The present study for the first time examines HDL associated vascular and inflammation associated miRs in healthy subjects and patients with stable CAD or ACS and elucidates to what extend HDL-miRs are transferred to endothelial cells. Moreover, we investigated the effects of HDL from healthy subjects and patients with coronary disease on miR levels that are relevant in the cardiovascular system in endothelial, smooth muscle and peripheral blood mononuclear cells.

We demonstrate that miR-223, miR-92a, and miR-126 were found at highest concentrations in HDL, whereas all other miRs tested were below 500 copy numbers/µg HDL. One has also to take into account that one µg HDL consists of a large amount of HDL-bound molecules, including proteins, lipids and small molecules. Therefore, we have to consider that likely not all HDL-bound molecules are binding miRs. However, our finding that miR-223 was the most abundant miR (about 10,000 copies/µg HDL) is consistent with the study of Vickers et al, showing that this miR is strongly enriched in purified HDL[14]. Overall, the concentration of HDL-miRs strongly correlated to the total plasma pool of circulating miRs raising the question of whether the presence of miRs in HDL might reflect unspecific binding to circulating miRs in the plasma. However, when calculating the percentage of HDL-miRs to the total pool of circulating miRs, we observed that some miRs such as miR-126, miR-378 and miR-223 were more efficiently bound to HDL as compared to other miRs (Fig. 1D) suggesting some specificity of the binding. The maximal levels, however, are still below 10 % of the respective plasma miR levels demonstrating that the majority of circulating miRs is not associated with HDL.

Moreover, we analyzed weather miRs are also associated with HDL`s counterpart LDL. LDL was shown to contain a different composition of miRs compared to HDL[14] and does not have the same functions, for instance is not able to activate endothelial nitric oxide synthase[33]. Similar to HLD, miR-223 is also the most abundant miR in LDL, but is reduced by 7-fold compared to HDL. Interestingly the only miR that is higher bound to LDL compared to HDL is miR-155 (177 copies/µg LDL vs. 44 copies/µg HDL). miR-155 promotes atherosclerosis and is specifically expressed in atherosclerotic plaques and proinflammatory macrophages[34]. Furthermore, LDL induces miR-155 expression in macrophages[34] indicating a possible transfer of LDL bound miR-155 to macrophages and atherosclerotic plaques respectively.

Our data further demonstrate that HDL-miRs are not efficiently taken up by endothelial cells. To determine the direct transfer of HDL-miRs to endothelial cells, we loaded HDL with recombinant *C. elegans* miRs and incubated these HDL-miR complexes with endothelial cells in vitro. We indeed detected the presence of *C. elegans* miRs in endothelial cells, however, the copy numbers are very low (<10 copies per cell). In lymphocytes, endogenous miRs were shown to be expressed at 5,000 – 33,000 copies per cell[35]. MiR-126 and miR-92a were found to be expressed in a similar range in our study (data not shown) suggesting that the low number of copies that are transferred by HDL are unlikely to have a significant biological function. Previous studies demonstrated that HDL delivers exogenous or endogenous miRs to SRBI expressing baby hamster kidney cells and hepatocytes, which are known to efficiently take up lipoproteins and oligonucleotides[36]. Endothelial cells were shown to express the SRBI receptor[37], but appear less sensitive for the up-take of HDL-bound miRs in our setting. In contrast, endothelial cells were shown to efficiently take up microvesicle-embedded miRs[6, 19]. Of note, we cannot exclude that differences in the HDL-preparations (e.g. Vickers et al. used recombinant HDL which was made free of RNA before loading) might have influenced the results. However, we controlled the loading of the HDL with *C. elegans* miRs ($1*10^7$-$6*10^9$ copies per µg HDL) and the incubation of endothelial cells with high concentrations of native HDL (up to 1 mg/ml) also did not increase the endogenous miRs in the cultured endothelial cells (Suppl. Fig. 4).

In contrast, native HDL tend to reduce the expression of miR-92a, miR-126 and miR-223. The HDL induced transient reduction of miRs was also observed in SMC and a continuous decline of miR expression in PBMCs. In addition, our data demonstrate that LDL did not increase the expression of the endothelial miRs excluding that LDL bound miRs, which are detected at even lower concentrations compared to HDL, are efficiently transferred (Suppl. Fig. 5).

Previous studies demonstrated that familial hypercholesterolemia is associated with a distinct HDL-miR pattern compared to normal subjects[14], however, the impact of cardiovascular disease was not explored. Therefore, we analyzed the miR profile of HDL that was purified from patients with stable CAD or ACS. Indeed, some miRs were significantly regulated in HDL from patients compared to normal subject. HDL-bound miR-92a was most profoundly reduced in CAD and ACS. Moreover, the inflammation associated miR-146 and miR-30c, whose function is unknown, were slightly but significantly down-regulated in HDL from ACS patients. Since no significant changes of LDL bound miRs were observed in patient derived LDL compared to LDL from healthy subjects LDL-miR pattern seems not to be regulated in cardiovascular disease. However, when incubating patient-derived HDL with endothelial cells, the transient reduction of endothelial miR levels induced by HDL from healthy subjects is abrogated. On the contrary, in smooth muscle cells and peripheral blood mononuclear cells the transient reduced miR expression caused by HDL from healthy subjects is even further intensified with patient derived HDL. Surprisingly, in PBMC a different effect is seen after longer incubation with HDL from patients with CAD or ACS. Here, miR-92a, miR-126 and miR-223 levels are increased. This increase was significantly different from what had been observed with normal subject derived HDL, which reduced these miRs. The different cellular responses to HDL derived from healthy controls versus patients are consistent with previous studies that documented a different biological activity of patient versus healthy control-derived HDL[30].

The rapid time course of miR expression changes suggest that HDL influences the turn over or processing of miRs. We discovered a differential regulated biogenesis of miR-126 compared to miR-92a and miR-223 in PBMC. The regulation of miR-126 levels appear to be primarily influence at the level of transcription, whereas the reduction of miR-92a and miR-223 is independent on a transcriptional change of the pri-miRs or a change in processing into the pre-miR. The reduction may be either due to a block of Dicer processing or a destabilization of the mature miR. An interesting alternative hypothesis to explain these findings may be that HDL removes miRs from cells. It is well known that HDL can be transported through endothelial cells[38] and can export lipids from atherosclerotic plaques, a process known as reverse cholesterol export[39]. However, we did not detect an increase in miRs in the supernatant of endothelial cells after incubation with HDL.

Interestingly, the effects on pri- and pre-miR expression after HDL incubation were similar in PBMC and endothelial cells (Suppl. Fig. 6). These results indicate a distinct regulation of different miRs by HDL. Further on, the different cellular responses to HDL from healthy subjects compared to patients derived HDL is also visible at the level of pri- and pre-miRs.

Together, our data confirm that miRs are transported in plasma by HDL and to a less part also by LDL. The contribution of HDL-bound miR to the total miR pool, however, is rather low. The HDL bound miRs were not efficiently delivered to endothelial, smooth muscle and peripheral blood mononuclear cells suggesting that the HDL associated pool of miRs is not of major importance for the regulation of the endothelial functions in these cells. However, the present data do not exclude that HDL-bound miRs may be taken up in vivo for instance in atherosclerotic lesions or foam cells.

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Figure legends

Figure 1

HDL, LDL and Plasma miR levels in healthy control subjects. miR expression determined by qPCR and copy numbers calculated from a standard curve for each miR with known concentrations. **A**, Detected copy numbers of indicated miRs per µg HDL (n=10). **B**, Detected copy numbers of indicated miRs per µg LDL (n=5) and HDL (n=10). **C,** Detected copy numbers of indicated miRs per ml plasma from the same individuals as HDL was isolated. **D**, Percentage of HDL bound miRs related to miRs detected in total plasma. **E**, Correlation between HDL bound miRs and plasma containing miRs (two-sided Spearman correlation). 10µl HDL resulted from 250µl plasma. **p<0.01 HDL versus LDL, ***p<0.001 HDL versus LDL (t-test with Welch´s correction); n.d.: not detected.

Figure 2

HDL bound miRs in patients with cardiovascular disease. miR expression determined by qPCR in HDL and plasma from control subjects (HS), patients with stable coronary artery disease (CAD) and acute coronary syndrome (ACS), n=10 each. Copy numbers calculated from a standard curve for each miR with known concentrations. **A**, Copy numbers of indicated miRs per µg HDL. **B**, Percentage of HDL bound miRs related to miR levels in total plasma. *p<0.05 and **p<0.01 versus HS (Anova with Bonferroni´s multiple comparison test).

Figure 3

HDL bound miR delivery to endothelial cells and effect of HDL on different cells. A, Experimental setup of HDL cel-miR-39 delivery experiments. HDL from healthy control subjects was incubated as indicated with artificial *C. elegans* miR (cel-miR-39), or two different double-stranded precursor miRs (cel-miR-39 mimic and cel-pre-miR-39) respectively, and applied to human umbilical venous endothelial cells (HUVEC). After 24 hours RNA was isolated, miRs detected by qPCR, and copy numbers calculated from a standard curve with known miR concentrations. **B**, Detected copy numbers per cell above background (HDL alone) in HUVEC incubated with HDL-cel-miR-39/mimic/precursor complex (HDL+miR), or cel-miR-39/mimic/precursor alone (miR), (n=4). **C**, Experimental setup of endogenously HDL bound miR delivery experiments. HDL from healthy control subjects was incubated with HUVEC (**D**), smooth muscle cells (SMC) (**E**) or peripheral blood mononuclear cells (PBMC) (**F**) for indicated time points, RNA isolated and miR expression determined by qPCR. Relative expression of miR-92a, miR-126 and miR-223 after addition (HDL) or no addition of HDL (co) for indicated time points, (n=4-14). Data were normalized to RNU6. *p<0.05 versus co, **p<0.01 versus co (t-test with Welch´s correction).

Figure 4

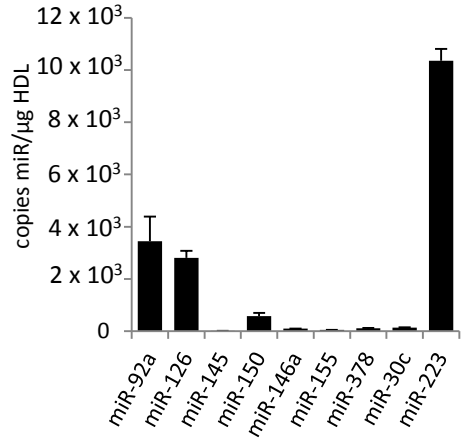
Influence of HDL from patients with cardiovascular disease on miR expression in different cells. Same experimental setup as shown in Figure 3C. Besides HDL from healthy subjects (HS), HDL form patients with stable coronary artery disease (CAD) and acute coronary syndrome (ACS) was incubated with HUVEC (n=10 each) (**A**), SMC (n=5 each) (**B**), and PBMC (n=5 each) (**C**). Data were normalized to RNU6. Relative expression of indicated miRs after addition (HDL) or no addition of HDL (-) for denoted time points. *p<0.05 HDL versus -, **p<0.01 HDL versus -, ***p<0.001 HDL versus -, $^{\#}$p<0.05 HDL CAD/ACS versus HDL HS $^{\#\#}$p<0.01 HDL CAD/ACS versus HDL HS, $^{\#\#\#}$p<0.001 HDL CAD/ACS versus HDL HS (Anova with Bonferroni´s multiple comparison test).

Figure 5

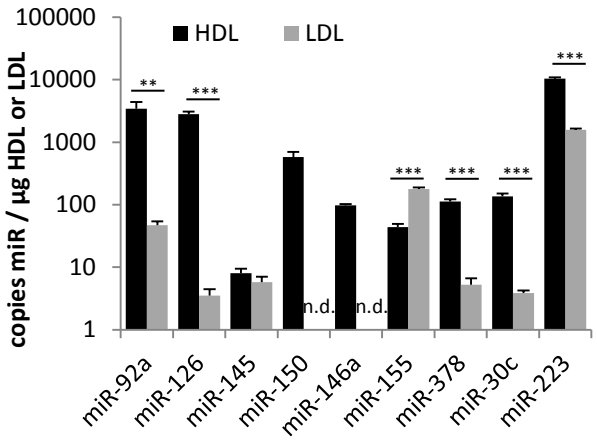
Influence of HDL on miR biogenesis in peripheral blood mononuclear cells
Same experiment as in Figure 4. **A**, Relative expression of primary (pri-) and precursor (pre-) miRs. **B**, Relative expression of Drosha and Dicer mRNAs. Data were normalized to ribosomal P0. *p<0.05 HDL versus -, **p<0.01 HDL versus -, ***p<0.001 HDL versus -, $^{\#}$p<0.05 HDL CAD/ACS versus HDL HS $^{\#\#}$p<0.01 HDL CAD/ACS versus HDL HS, $^{\#\#\#}$p<0.001 HDL CAD/ACS versus HDL HS (Anova with Bonferroni´s multiple comparison test).

Figure 1

A

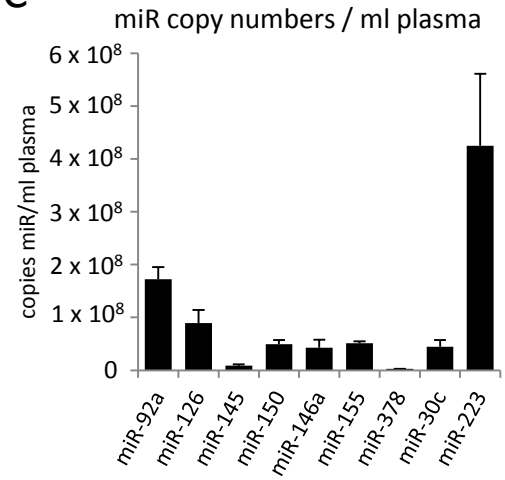


B

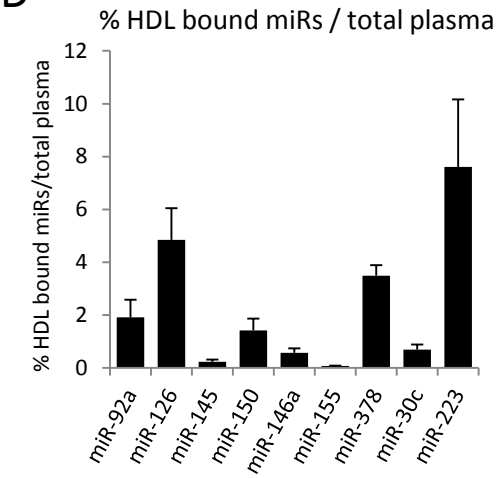


	HDL	LDL
miR-92a	3441	47
miR-126	2802	4
miR-145	8	6
miR-150	580	n. d.
miR-146a	97	n. d.
miR-155	44	177
miR-378	112	5
miR-30c	136	4
miR-223	10358	1570

C



D



E

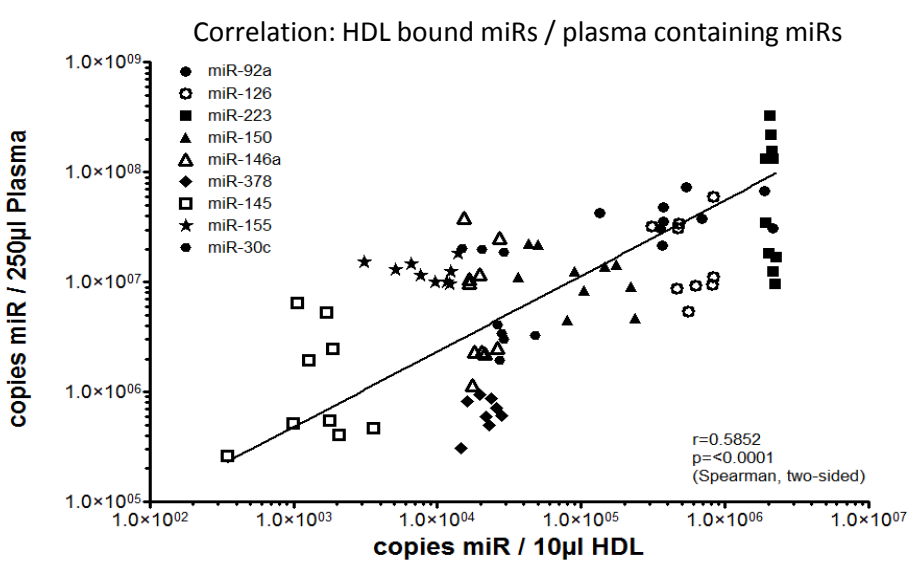
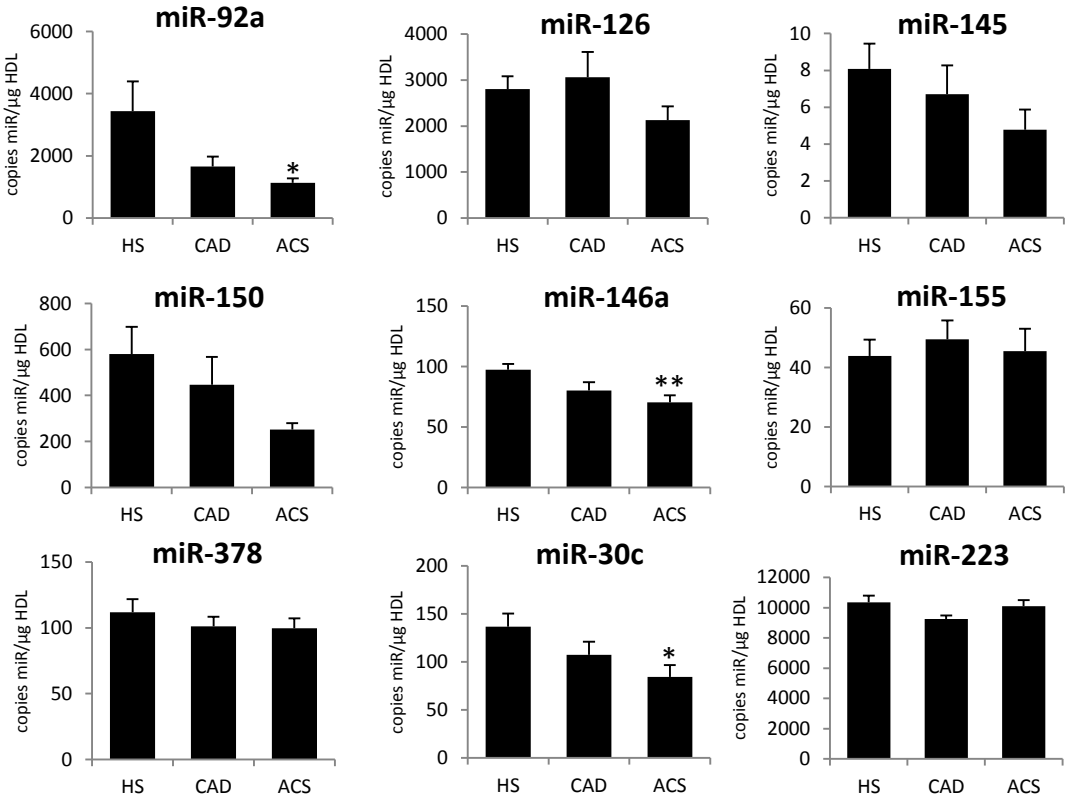


Figure 2

A Copy numbers / µg HDL



B % HDL-bound miRs

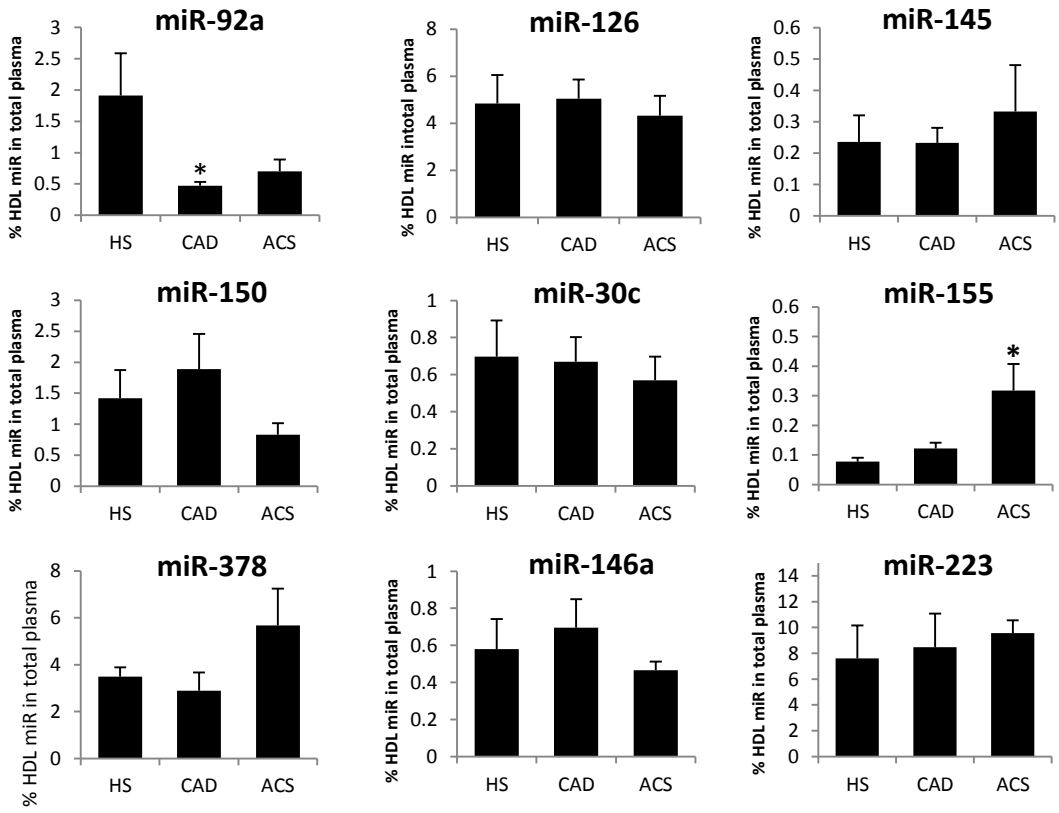


Figure 3

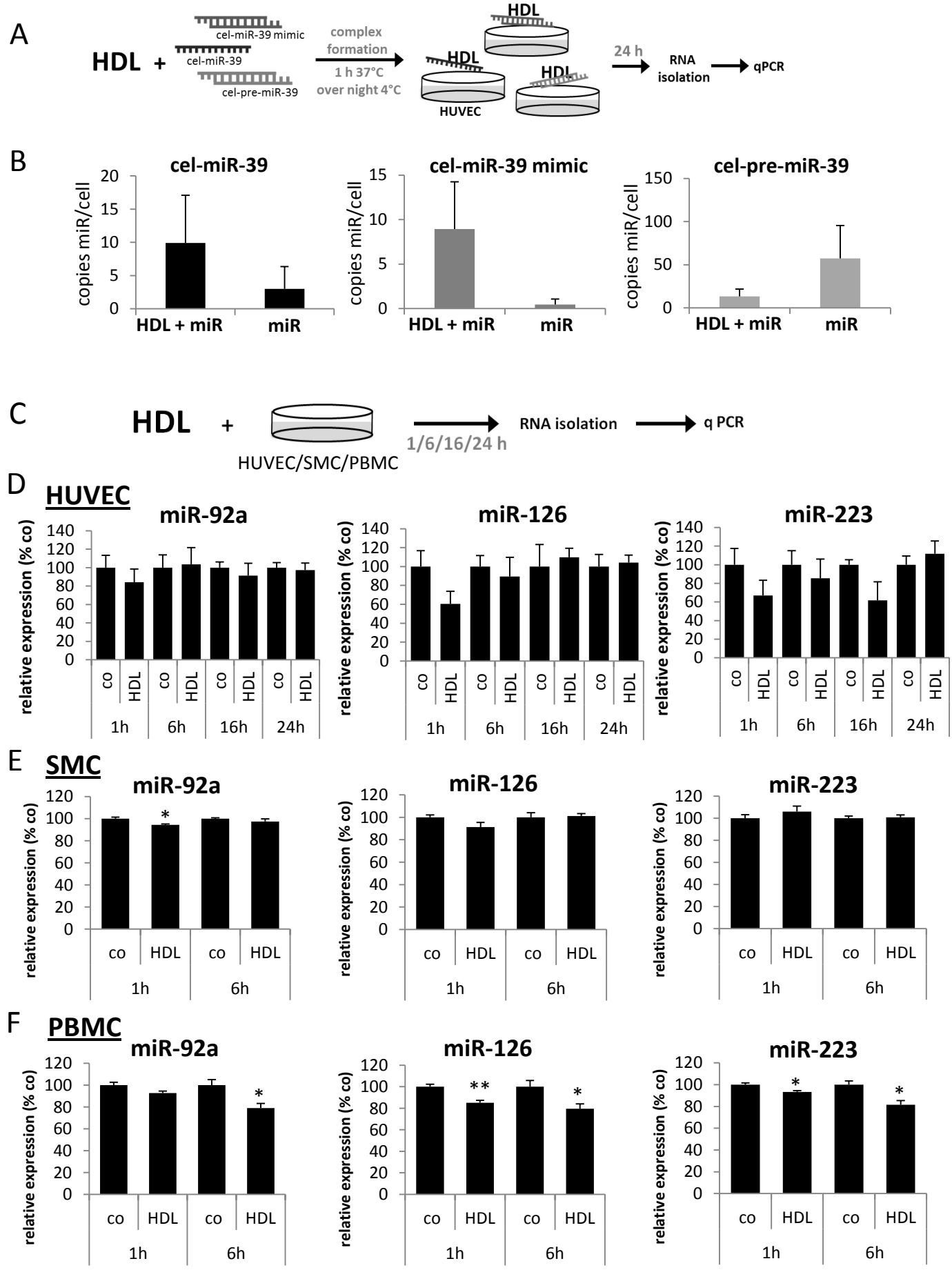

A
HDL +
cel-miR-39 mimic
cel-miR-39
cel-pre-miR-39
complex formation
1 h 37°C
over night 4°C
HDL
HDL
HDL
HUVEC
24 h
RNA isolation
qPCR
B
cel-miR-39
cel-miR-39 mimic
cel-pre-miR-39
copies miR/cell
HDL + miR
miR
C
HDL +
HUVEC/SMC/PBMC
1/6/16/24 h
RNA isolation
q PCR
D
HUVEC
miR-92a
miR-126
miR-223
relative expression (% co)
co
HDL
1h
6h
16h
24h
E
SMC
miR-92a
miR-126
miR-223
F
PBMC
miR-92a
miR-126
miR-223

Figure 4

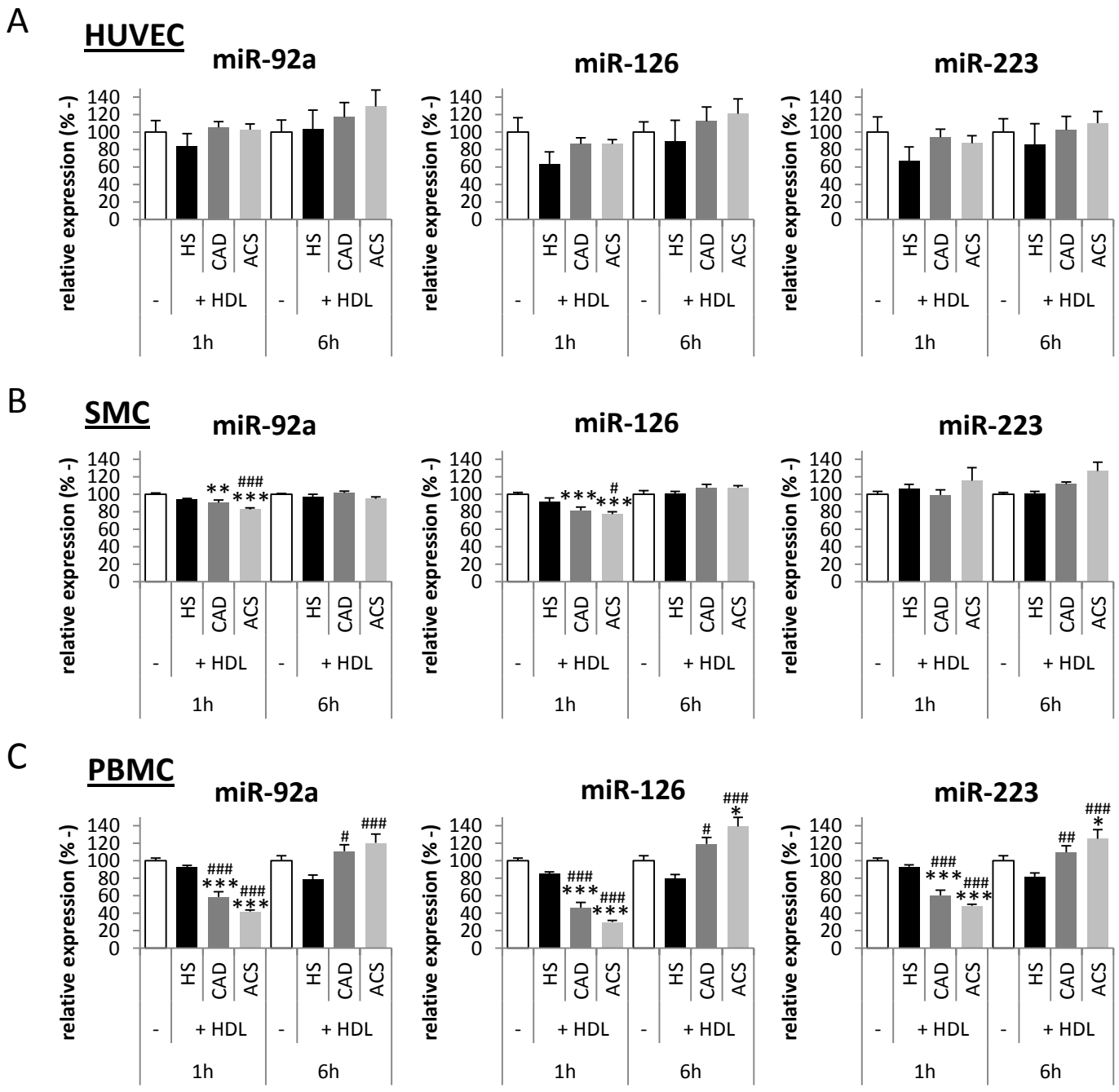
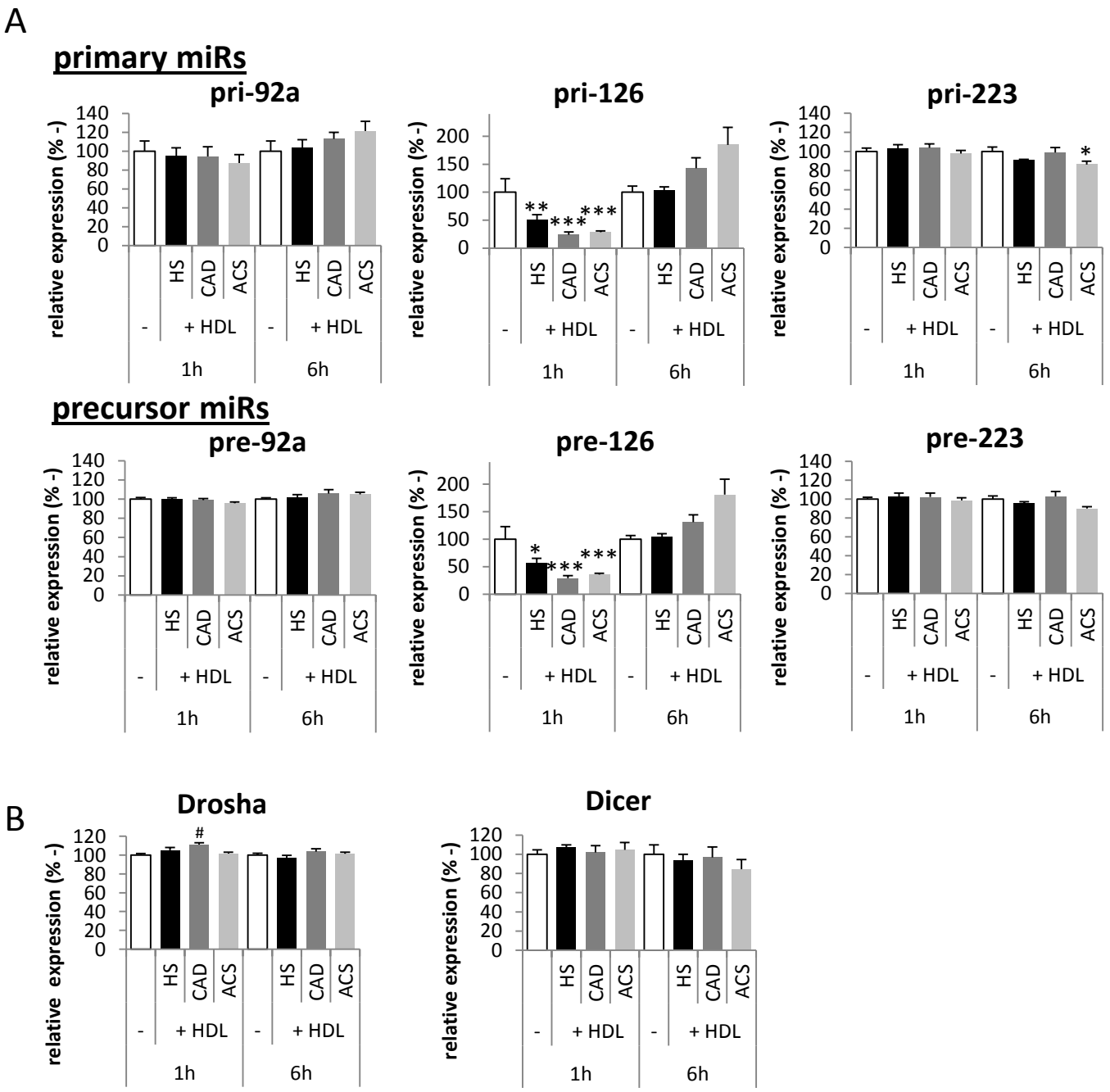

A
HUVEC
miR-92a
miR-126
miR-223
B
SMC
miR-92a
miR-126
miR-223
C
PBMC
miR-92a
miR-126
miR-223
relative expression (% -)
HS
CAD
ACS
-
+ HDL
1h
6h

Figure 5


A
primary miRs
pri-92a
pri-126
pri-223
precursor miRs
pre-92a
pre-126
pre-223
relative expression (% -)
HS
CAD
ACS
-
+ HDL
1h
6h
B
Drosha
Dicer
relative expression (% -)

Response to reviewers

Reviewer #1:

MAJOR POINTS:

1) "Incubation of endothelial cells (EC) with native HDL significantly reduced the endogenous expression of miR-223, miR-92a and miR-126 suggesting that HDL reduces the biosynthesis or enhances the export of miRs from EC.": To get a more insights in these hypotheses, Authors should measure primiR and premiR forms of the selected miRs as well as Drosha and Dicer in cultured ECs stimulated with endogenous HDL. On the other side, they should incorporate fluorescent miRs in cultured ECs and assess if HDL influence the release of these and if so by what mechanisms (for example, exosome release by specifically blocking this at some stage). Because Authors noticed that " HDL from patients with CAD did not reduce these endothelial miRs but rather induced a significant up-regulation of endothelial miRs", the aforementioned experiments should be performed using as stimulus HDL from either healthy or diseased subjects.

In order to address this question, further experiments with HDL from more healthy subjects were performed. The effect of transient miR down regulation is no longer significant, only a trend is observable. Nevertheless, primary and precursor miR as well as Drosha and Dicer mRNA levels were measured after treatment of endothelial cells with HDL in different concentrations (n=4). No significant effects on miR biosynthesis were observed (Suppl. Figure 6, revised manuscript). In addition, we analyzed the influence of HDL on miR biogenesis in PBMC in more detail and included these data in the revised manuscript (Figures 4 and 5, revised manuscript). The effects in PBMC reflect the tendencies observed in endothelial cells
To analyze the possible release of miRs, we measured miR expression in supernatants of HUVEC after incubation with HDL. However, we did not see any increase in miR in the supernatant of cells exposed to HDL. These data may suggest that the minor reduction of miRs in the cells is most likely due to an enhanced intracellular degradation of mature miRs.

2) Authors should study the functional impact of stimulating ECs with HDL-conjugated miRs from healthy and disease patients. Here, some in vitro EC biology assay should be performed.

We performed aortic ring and tube formation assays (Figure 1 for the reviewers). In the aortic ring assay intensive sprouting was observed after incubation with HDL from healthy subjects whereas strongly reduced sprouting was visible after incubation with patient derived HDL (CAD and ACS). In addition, a decreased tube formation capacity of endothelial cells was monitored when cells were incubated with HDL from patients with coronary disease in contrast to HDL from healthy subjects (Figure1 for the reviewers).

3) Authors should additional investigate SMCs and monocytes as targets of HDL-miRs. In other words, the experiments should not only focus on ECs, but expand to other relevant cell types.

According to the suggestion of the reviewer, we have performed additional extensive studies in SMCs and peripheral blood mononuclear cells (PBMC). Interestingly, the effect of HDL on miRs was distinct in the different cell types. In SMC, miR-92a and miR-126 were slightly reduced after 1 hour incubation with HDL from healthy subjects (Fig. 3E, revised manuscript) and further reduced after incubation with patient derived HDL (Fig. 4B, revised manuscript). miR-223 expression is not significantly changed after HDL treatment in SMC (Fig. 3E and 4B, revised manuscript). Most

profound changes were observed in HDL-treated PBMC. Treatment with HDL from healthy subjects reduced the levels of miR-126 and miR-223 by about 15%. miR-92a is only slightly reduced after 1 hour, but also significantly reduced after 6 hours incubation with HDL from healthy volunteers (Fig. 3F, revised manuscript). In contrast, all three miRs are reduced by more than 60% after incubation with HDL from patients with CAD, and more than 50% after treatment with HDL from patients with ACS for 1 hour (Fig. 4C, revised manuscript). On the contrary 6 hours after incubation of PBMC with HDL from patients with coronary disease all analyzed miRs are significantly increased again compared to treatment with HDL from healthy subjects (Fig. 4C).
These data have been included in the revised manuscript on page x, para y and Figure 3 and 4.

MINOR POINTS

1) The title is very broad, but the focus of the paper is not. Title should be more specific and related to the experiments developed in the study.

New title? (will have to think about… bin nicht sicher)

2) The Authors should describe or speculate if other form of lipoprotein carry miRs and what are the processes leading to the appearance of HDL-miR in the circulation. Are cells releasing miRs as already conjugated with HDL? Is the encounter happening in the peripheral blood? Please, provide more info or speculations.

According to the suggestion of the reviewers, we measured the miR content of other lipoproteins such as LDL. Interestingly, we observed a significant change of nearly all miRs when comparing miR copies bound to HLD vs. LDL (Fig. 1B, revised manuscript). miR-223 is also highest abundant in LDL, but only 1,500 copies/µg LDL compared to <10,000 copies/µg HDL miR-223 were detected. The concentrations of most LDL-bound miRs (miR-126, miR-145, miR-150, miR-378 and miR-30c) were extremely low (<10 copies /µg LDL). In contrast the pro-atherosclerotic miR-155 is higher abundant in LDL compared to HDL.
No significant differences in miR levels isolated from healthy subjects compared to LDL from patients with CAD and ACS could be detected (Suppl. Fig. 2, revised manuscript).Treatment of endothelial cells with LDL revealed no influence on endothelial miR expression (Suppl. Fig. 5, revised manuscript).
These data are included in the revised manuscript on page x, para y and Figure 1 and Suppl. Figures 2 and 5.

3) Correlation of Figure 1D (plasma miRs vs HDL-miRs in healthy subjects) should be repeated in the patient populations. Also, Figure 1D. I understand that they wish to demonstrate that miRs that are generally at a higher concentration in plasma are also generally at a higher concentration in HDL. But it is also clear from the scatterplot that some miRs had an almost constant expression in HDL despite a wide range of plasma concentrations, e.g. miR-223, miR-145, miR-378, miR-146a. This is worth mentioning.

In Figure 1E we show that HDL-bound miRs significantly correlate with plasma miRs. However, we admit that miR-223, miR-146a and miR-378 show a relatively constant expression in HDL and on the other hand vary in the plasma of individual subjects. On the other hand miR-155 and miR-92a expression differs in HDL and is more constant in plasma.
Patient populations also show a significant correlation of HDL-bound miRs and plasma miRs (Suppl. Figure 1, revised manuscript). However, consistent with the data obtained in healthy controls, miR-223 and miR-378 are relatively constant expressed in HDL and differ in plasma. According to the

suggestion of the reviewer, we mentioned this aspect in the revised manuscript (page x, para y) and included the correlation analysis in patients as supplementarl Figure 1.

4) Details on ethical approval for taking the patient samples (and sending to Frankfurt) are missing

Ulf and Meliana, could you please help to answer this question?

5) When describing the different cel-miRs added to HDL, it is worth giving the trademark names for the 'mimic' and 'precursor', especially as they give different results later on. How was the 'mature' cel-miR made/purchased?

According to the suggestion of the reviewer, we added further information in the manuscript as follows:
Ambion Pre-miR Custom Precursor miRNA cel-miR-39 (Life Technologies)
mirVana miRNA mimic cel-miR-39 (Life Technologies)
mature cel-miR is an RNA oligonucleotide consisting of the sequence of mature cel-miR-39 according to Sanger miRBase (purchased from Sigma Aldrich)

6) When giving the HDL protein concentration, is this the concentration in the serum or in the 10ul isolated fraction? I would have initially assumed it was the former, and I only ask because I thought that normal HDL concentration was much lower than this (0.35 - 1.35 ug/ul)

18-25 µg/µl is the protein concentration in 10 µl HDL concentrate generated from 250 µl serum and thus reflects the concentration of 0.72 – 1 µg HDL/µl serum. We added this information to the revised manuscript.

7) For reverse transcription it is stated that '10ug total RNA' was used. Do they mean 10ng?

We thank the reviewer for noting this mistake. We used 10 ng RNA and accordingly changed the text of the revised manuscript.

8) Where are the 'recombinant miRs' used for the standard curve from?

The recombinant miRs are RNA oligonucleotides, purchased from Sigma Aldrich. This information was added in the revised manuscript.

9) There is no mention of a spiked-in exogenous miR when extracting RNA. How was extraction efficiency tested? If the extraction efficiency is <

For the experiments shown in Figure 1 and Figure 2 cel-miR-39 was used as a spike in control (RNA Oligonucleotide from Sigma-Aldrich). For the calculations of miR copy numbers unnormalized values were used. However cel-miR-39 normalized and unnormalization results are comparable. This information was added in the revised manuscript.

11) Figure 1A and 1B. Need to change y-axis to scientific notation - 1x10n

As recommended by the reviewer, we changed the y-axis.

12) Page 8, first line. They mention cel-pre-miR here. Was this a double-strand oligo without a stem-loop structure or a true premiR?

The precursor miR and the mimic are both double-stand RNA oligonucleotides without a stem-loop structure. The difference between both oligos is the stabilizing chemistry (correspondence with Life Technologies).

13) Can the authors think of a reason why the HDL-free miR 'premiR' and 'mimic' had very different levels of incorporation into the endothelial cells?

The reason for the different uptake of precursor and mimic might be due to the different stability due to the different chemistry of both oligos.

*14) Figure 2B, miR92a graph. There is no * above the ACS bar but in the text it says there was a significant reduction.*

The sentence in the text was changed to make the result more clear.

*15) Figure 4. For the 1hr HDL bars there is no * above them for miR92a (CAD), miR126 (CAD) and miR-126 (ACS). Are these definitely not significant differences vs. control?*

Shown are the results of ttest with welch´s correction, there is no significance for the mentioned groups (CAD miR-92a 1h: p=0.0883; CAD miR-126 1h: p=0.1409; ACS miR-126: 1h p=0.0504).

16) The CAD and ACS populations from Fig 4 are also on primary/secondary prevention medications (e.g. nearly all on statins vs. none of the healthy subjects). Is there any evidence in the literature that these can have an effect on HDL function?

Ulf and Meliana, could you please help to answer this question?

17) Supplementary Figure 1: Some error bars are missing in B and D for the control conditions (unless all replicates were exactly the same!). Again, check that the differences in panel B vs control aren't significant. If there was genuinely hardly any standard error in the control condition replicates, then both of these should be significantly different to the pre-223 conditions. Missing scale bars in panels C and E

For each experiment in Figure B and D (Suppl. Figure 3, revised manuscript) control was set to 100%, therefore there are no error bars for the controls. T-test p-values for Figure 1B are p=0.707 for 1 nM precursors and p=0.789 for 10 nM precursors.
Scale bars were added in C and E.

18) There are quite a few minor spelling/formatting errors (e.g. 'ASC' instead of 'ACS', or using decimal points instead of a comma for large numbers).

We corrected these errors.

Reviewer #2:

Major comments

1. How many copies of cel-miR-39, cel-miR-39 mimic, and cel-pre-miR-39 were bound to HDL? Was there a difference in the binding affinity between the oligonucleotides?

Only a slight difference in binding affinity of the different RNA oligonucleotides was observed (Cel-miR-39: 5.6 x 10^9 copies/µg HDL, cel-miR-39 mimic: 1.2 x 10^7 copies/µg HDL, cel-pre-miR-39: 2.8 x 10^8 copies/µg HDL). These data are discussed in the discussion section of the revised manuscript.

2. Were the copy numbers of non-HDL bound miRNAs controlled for the amount of HDL bound oligos added to HUVECs?

We admit that this was not controlled.

3. Previous studies have demonstrated that miRNAs miR-92a, miR-126, and miR-155 are decreased in the plasma of CAD patients. Interestingly, HDL bound miR-126 and miR-155 levels were not different or rather increased in patient samples in this study. What were the total plasma levels of these miRNAs?

As shown in Figure 2 for the reviewer, miR-126 and miR-155 are both reduced in plasma of CAD and ACS patients These data suggest that the reduction of miR-126 and miR-155 in total plasma is not related to difference in HDL binding activity.

4. In the Supplemental Table 1 depicting study population characteristics, surprisingly, CRP levels seem to be significantly higher in healthy subjects compared to ACS patients. Could this be due to a selection bias? If so, this could have influenced the data regarding inflammation-associated miRNAs as well. Please comment.

We thank the reviewer for raising this point. There was a mistake in the statistical analysis. After rechecking there is no significant change in CRP levels (one-way Anova).

5. In the same Supplement Table 1, p-values indicate that there is a significant difference in HbA1c values, but the HbA1c numbers do not reflect any difference. On other hand, in Supplement Table 2, there appears to be some difference in HbA1c levels between the 3 gp, but no significance is reported.

There was a mistake in the statistical analysis. After rechecking there is no significant change in HbA1c numbers in supplement Table 1 (one-way Anova). The differences in HbA1c levels in Suppl. Table 2 are not significant (p-value 0.0692, one-way Anova).

6. In the Supplementary figure 1, were sprout formations significantly increased with miR-223 up-regulation?

Results are not significant, T-test p-values are p=0.707 for 1 nM precursors and p=0.789 for 10 nM precursors.

7. The data shown in the Supplement are not sufficient to conclude that miR-223 is an angiogenic regulator in endothelial cells. Is miR-223 down regulated in the endothelium of CAD patients? Did miR-223 inhibition have any effect on endothelial cell angiogenesis?

Unfortunately, it is not possible to isolate the endothelium of patients to check miR-223 expression. We have to admit that miR-223 is only very low expressed in cultured endothelial cells therefore an effect on angiogenesis could only be discovered by overexpressing this miR. Therefore, the angiogenic activity of endogenous miR-223 is unclear. Accordingly, we modified the result section (page x, para y).

Minor comments

1. Page 9, 'Figure 4C' should be 'Figure 1C'.
2. Please check the manuscript for typos.

We corrected these errors.

Reviewer #3:

Described effects are rather minor and in the present form the manuscript does not provide their mechanistic explanation.

Major points:

1. What is the mechanisms of transient down regulation of endothelial microRNAs by HDL-bound microRNAS? And, oppositely, the mechanisms responsible for the transient increase in microRNAs when ECs were incubated with HDLs from CAD patients? This has not been addressed.

In order to address this question, further experiments with HDL from more healthy subjects and patients with coronary disease were performed. However, the effect of transient miR down regulation by HDL from healthy subjects is no longer significant, only a trend is observable. The same applied also for the increase of miR levels by patient derived HDL. Nevertheless, primary and precursor miRs as well as Drosha and Dicer mRNA levels were estimated after treatment of endothelial cells with HDL in different concentrations (n=4). No significant effects on miR biosynthesis could be observed (Suppl. Figure 6, revised manuscript). However, we analyzed the influence of HDL on miR biogenesis in PBMC in more detail and included these data in the revised manuscript (Figure 4 and 5, revised manuscript). The effects in PBMC reflect the tendencies observed in endothelial cells.

2. Did those changes translate to the regulation of the expression of mRNA targets of those transferred microRNAs?

To address the question of the reviewer, we elucidated the effect of HDL on miR targets. However, consistent with the minor and transient down-regulation of miR levels, miR targets were not regulated even when increasing the concentration of HDL to 1 mg/ml (Figure 3 for the reviewer).

3. The authors tested, using spheroid and Matrigel assay, the pro-angiogenic effect of miR-223 shown in Suppl Fig 1). However, as this miRNAs is the most abundant in HDLs, the effect of HDLs from healthy specimens and patients on such processes should be also investigated.

We performed aortic ring and tube formation assays (Figure 1 for the reviewers). In the aortic ring assay intensive sprouting was observed after incubation with HDL from healthy subjects whereas strongly reduced sprouting was visible after incubation with patient derived HDL (CAD and ACS). A decreased tube formation capacity of endothelial cells was monitored when cells were incubated with HDL from patients with coronary disease in contrast to HDL from healthy subjects.
However, we believe that these angiogenesis-regulating effects of HDL are independent on the regulation of miR-223 since no up-take was observed in endothelial cells under any conditions.

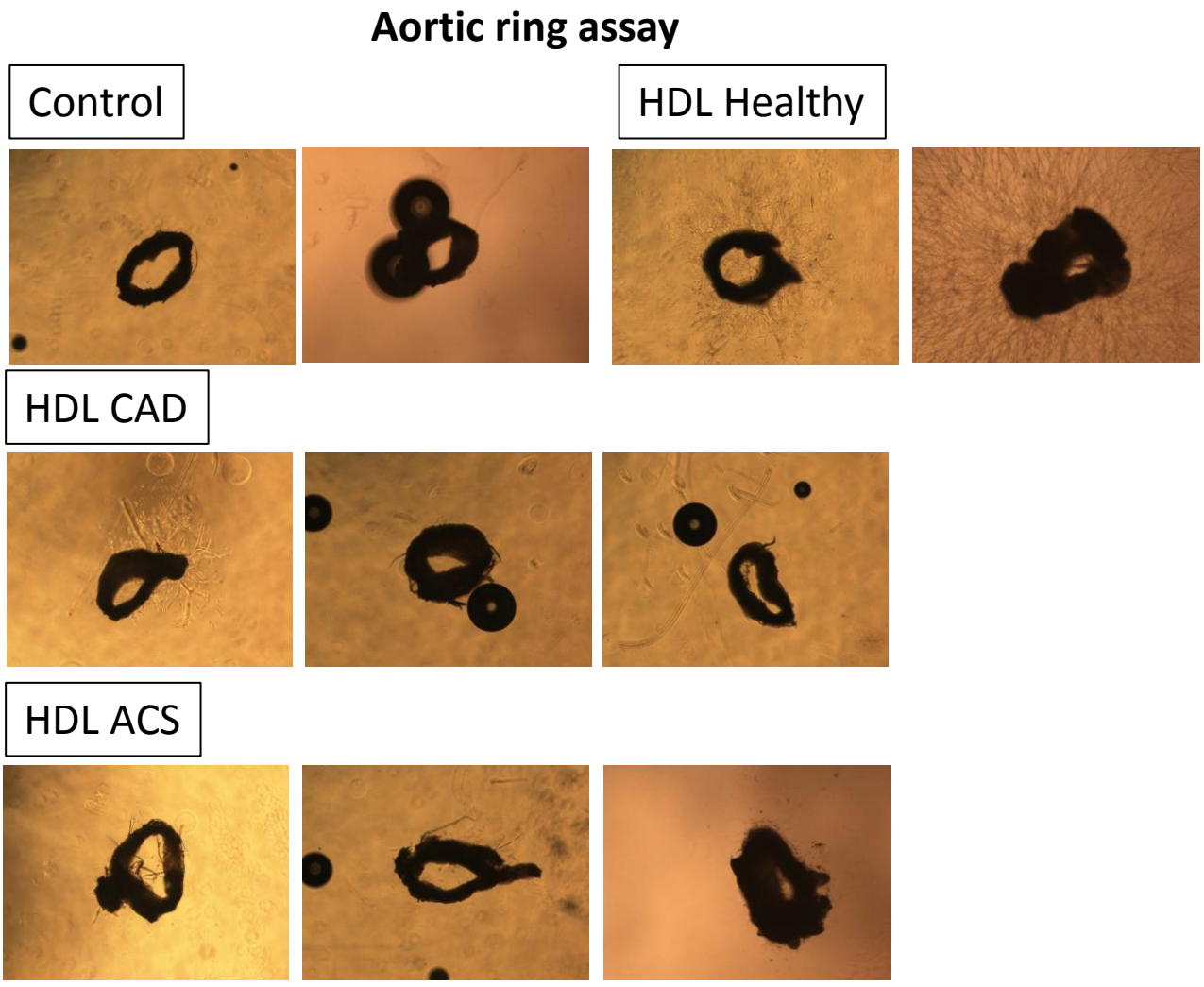
Other comments

1. The sentence on HDL isolation has to be corrected (the words „sequential ultracentrifugation" have been used twice)

2. The way of writing numbers and following SI units/other units is not correct. There should be a space between the number and unit (eg. should be "20 pg" not "20pg")

These mistakes have been corrected.

Figure 1 for the reviewers



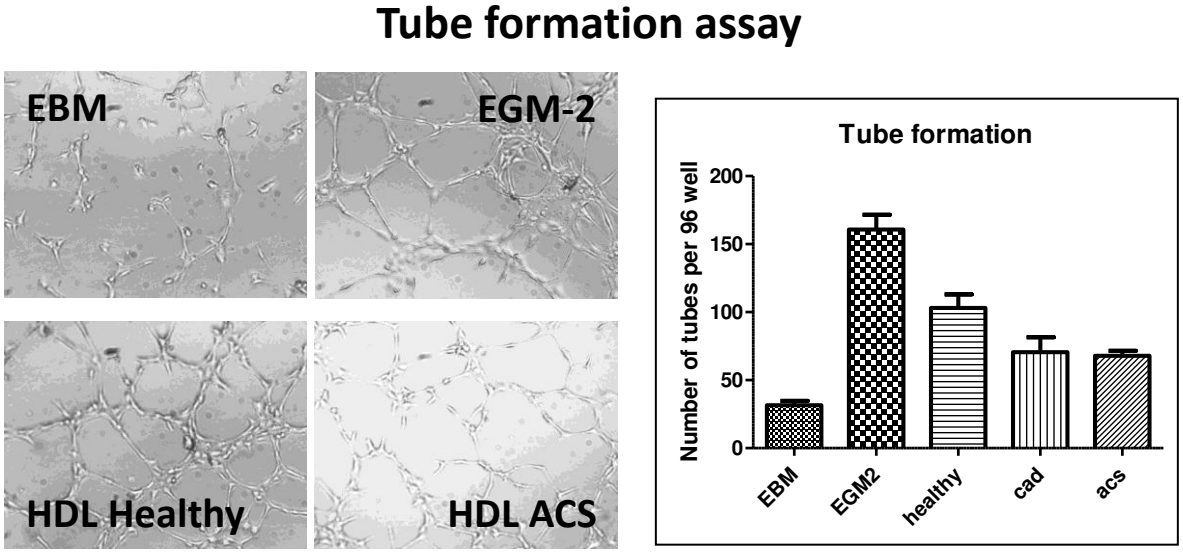


Figure 2 for the reviewers

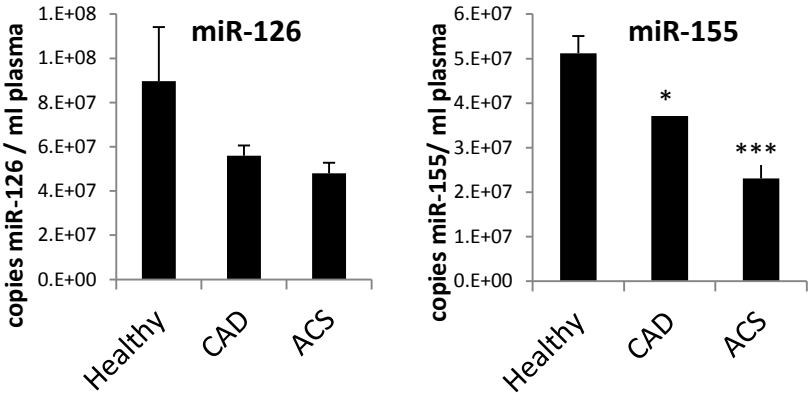
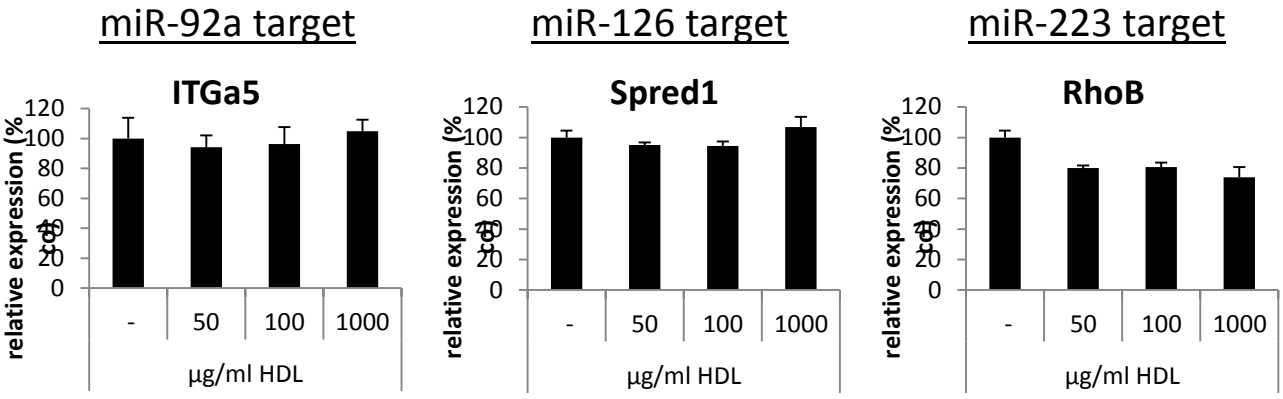


Figure 3 for the reviewers



SUPPLEMENTAL MATERIAL

Supplemental Methods

Spheroid Assay

Cell spheroids of HUVEC were generated as described previously [1, 2]. In vitro angiogenesis was quantified by measuring the cumulative length of all sprouts of each spheroid using a digital imaging software (AxioVision Rel. 4.8, Carl Zeiss). 10-12 spheroids per group and experiment were analyzed.

Vascular Network Formation

48 hours after transfection 75,000 HUVEC per well were cultured in 12 well plate coated with 200µl Matrigel Matrix Basement Membrane Matrix (BD Biosciences). Tube length was quantified after 24 hours by measuring the cumulative tube length in three random microscopic fields using a digital imaging software (AxioVision Rel. 4.8, Carl Zeiss).

Primer Sequences for qRT-PCR

The following Primer sequences were used: P0for 5´-TCGACAATGGCAGCATCTAC-3´; P0rev 5´-ATCCGTCTCCACAGACAAGG-3´; pri-miR-17-92for 5´-CCAATAATTCAAGCCAAGCAA-3´; pri-miR-17-92rev: 5´-AAATAGCAGGCCACCATCAG-3´; pre-miR-92for 5´-TCTACACAGGTTGGGATCGG-3 ; pre-miR-92rev 5´-CGGGACAAGTGCAATACCATA-3´; pri-miR-126for 5´-GCCTCATATCAGCCAAGAAGG-3´; pre-miR-126for 5´-TGGCGACGGGACATTATTAC-3´; pri/pre miR-126rev 5´-GGACGGCGCATTATTACTCA-3´; pri-miR-223for 5´-GGGTGTGACTTCATCATTCC-3´; pre-miR-223for 5´-CCTCCTGCAGTGCCACGC-3´; pri/pre miR-223rev 5´-GCATGTGCCGCACTTGGGGT-3´. For detection of Dicer and Drosha mRNA QuantiTect Primer Assays (Qiagen) were used.

Supplemental Table 1

Characteristics of the study population in Figure 2 (HDL).

Characteristics	Healthy Subjects n = 10	Stable coronary artery disease n = 10	Acute coronary syndrome n = 10	P-value
Demographics				
Age, mean (years)	54	57	54	n.s
Sex (male/female)	6/4	6/4	6/4	
BP systolic, mean (mm Hg)				
BP diastolic, mean (mm Hg)				
MAP, mean (mm Hg)	98.1 ± 1.9	98.9 ± 4.7	98.2 ± 4	n.s
BMI, mean (kg/m^2)	25 ± 0.6	26 ± 0.9	25 ± 0.4	n.s
Laboratory parameters				
Glucose (mmol/l)	4.9 ± 0.1	5.7 ± 0.2	5.3 ± 0.3	<0.05
HbA1c (in %)	5.4 ± 0.05	5.5 ± 0.17	5.4 ± 0.07	n.s
Total cholesterol (mmol/l)	4.9 ± 0.3	4.3 ± 0.4	4.5 ± 0.2	n.s
HDL cholesterol (mmol/l)	1.6 ± 0.2	1.3 ± 0.1	1.2 ± 0.1	n.s
LDL cholesterol (mmol/l)	2.7 ± 0.3	2.7 ± 0.3	2.9 ± 0.2	n.s
Triglyceride (mmol/l)	1.0 ± 0.1	1.2 ± 0.1	1.2 ± 0.1	n.s
CRP (µmol/l)	2.0 ± 0.7	1.9 ± 0.6	1.7 ± 0.3	n.s
Creatinine (µmol/l)	85 ± 2	83 ± 5	83 ± 4	n.s
Medications				
Statins (in %)	0	70	40	

Abbreviations: BP, blood pressure; BMI, body mass index; MAP, mean arterial pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CRP, C-reactive protein; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker.
Reported P values are from one-way ANOVA.

Supplemental Table 2

Characteristics of the study population in Figure 4 (SMC/PBMC) and Suppl. Figure 1 (LDL)

Characteristics	Healthy Subjects	Stable coronary artery disease	Acute coronary syndrome	P-value
	n = 5	n = 5	n = 5	
Demographics				
Age, mean (years)	58	63	52	n.s
Sex (male/female)	3/2	4/1	3/2	
BP systolic, mean (mm Hg)	116 ± 6	131 ± 14	130 ± 20	n.s
BP diastolic, mean (mm Hg)	71 ± 9	77 ± 9	81 ± 10	n.s
MAP, mean (mm Hg)	86 ± 7	95 ± 10	97 ± 13	n.s
BMI, mean (kg/m^2)	22 ± 3	26 ± 3	25 ± 4	n.s
Laboratory parameters				
Glucose (mmol/l)	4.8 ± 0.4	5.4 ± 0.5	6.7 ± 0.8	<0.01
HbA1c (in %)	5.6 ± 0.1	5.7 ± 0.6	5.9 ± 0.3	n.s
Total cholesterol (mmol/l)	5.4 ± 0.3	4.4 ± 1	5.4 ± 0.3	n.s
HDL cholesterol (mmol/l)	1.8 ± 0.4	1.5 ± 0.5	1.2 ± 0.3	n.s
LDL cholesterol (mmol/l)	2.9 ± 0.2	2.4 ± 0.6	3.7 ± 0.9	<0.05
Triglyceride (mmol/l)	0.7 ± 0.1	1.2 ± 0.8	1.0 ± 0.4	<0.05
CRP (µmol/l)				
Creatinine (µmol/l)	80 ± 3	79 ± 4	66 ± 9	<0.01
Medications				
Statins (in %)	0	80	0	
Beta blocker (in %)	0	80	0	
Diuretics (in %)	0	60	0	
ACE-I/ARB (in %)	0	0	0	
Calcium blocker	0	40	0	
Aspirin (in %)	0	80	0	
Clopidogrel (in %)	0	80	0	

Abbreviations: BP, blood pressure; BMI, body mass index; MAP, mean arterial pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CRP, C-reactive protein; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker. Reported P values are from one-way ANOVA.

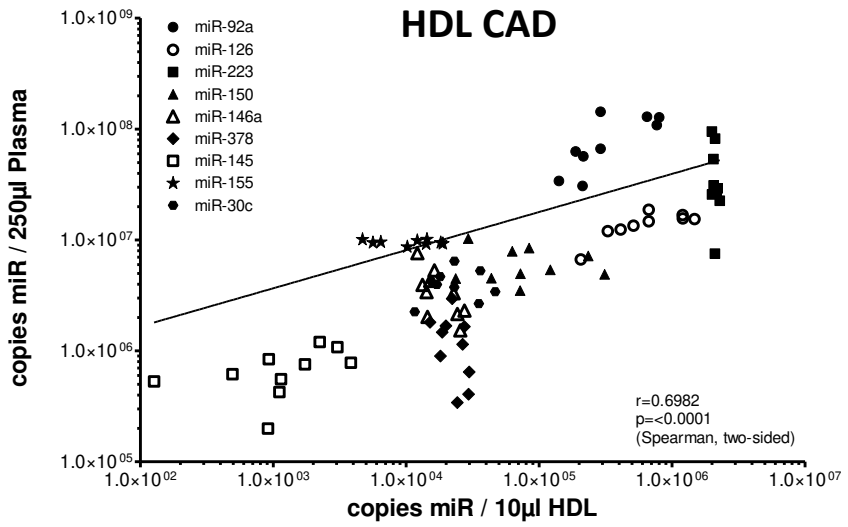
Supplemental Table 3

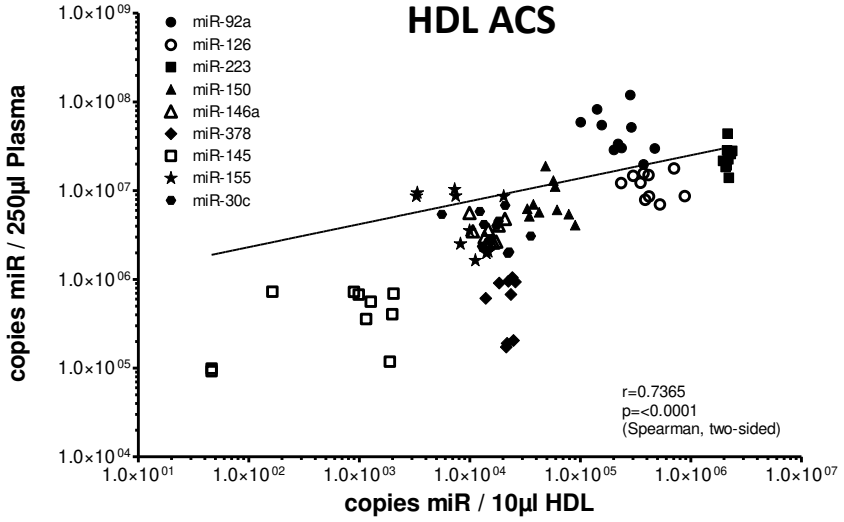
Characteristics of the study population in Figure 4 (HUVEC).

Characteristics	Healthy Subjects n = 10	Stable coronary artery disease n = 10	Acute coronary syndrome n = 10	P-value
Demographics				
Age, mean (years)	60	60	58	n.s
Sex (male/female)	6/4	7/4	6/4	
BP systolic, mean (mm Hg)	118 ± 10	127 ± 13	127 ± 21	n.s
BP diastolic, mean (mm Hg)	74 ± 9	80 ± 8	77 ± 13	n.s
MAP, mean (mm Hg)	88 ± 8	96 ± 8	94 ± 15	n.s
BMI, mean (kg/m^2)	22 ± 3	28 ± 3	27 ± 5	<0.01
Laboratory parameters				
Glucose (mmol/l)	4.8 ± 0.7	5.4 ± 0.4	6.8 ± 1.1	<0.001
HbA1c (in %)	5.5 ± 0.3	5.5 ± 0.6	6.0 ± 0.3	n.s
Total cholesterol (mmol/l)	5.4 ± 0.6	4.3 ± 0.9	4.9 ± 1.1	<0.05
HDL cholesterol (mmol/l)	1.8 ± 0.4	1.3 ± 0.4	1.3 ± 0.3	<0.01
LDL cholesterol (mmol/l)	3.2 ± 0.5	2.4 ± 0.7	3.0 ± 1.1	n.s
Triglyceride (mmol/l)	0.9 ± 0.3	1.3 ± 0.6	1.4 ± 0.7	n.s
CRP (µmol/l)	0.8 ± 0.3	4.2 ± 6	4.0 ± 3.6	n.s
Creatinine (µmol/l)	79 ± 8	88 ± 17	67 ± 11	<0.01
Medications				
Statins (in %)	0	90	40	
Beta blocker (in %)	0	80	20	
Diuretics (in %)	0	30	10	
ACE-I/ARB (in %)	0	50	50	
Calcium blocker	0	20	10	
Aspirin (in %)	0	90	30	
Clopidogrel (in %)	0	60	30	

Abbreviations: BP, blood pressure; BMI, body mass index; MAP, mean arterial pressure; HDL, high-density lipoprotein; LDL, low-density lipoprotein; CRP, C-reactive protein; ACE-I, angiotensin-converting enzyme inhibitor; ARB, angiotensin receptor blocker. Reported P values are from one-way ANOVA.

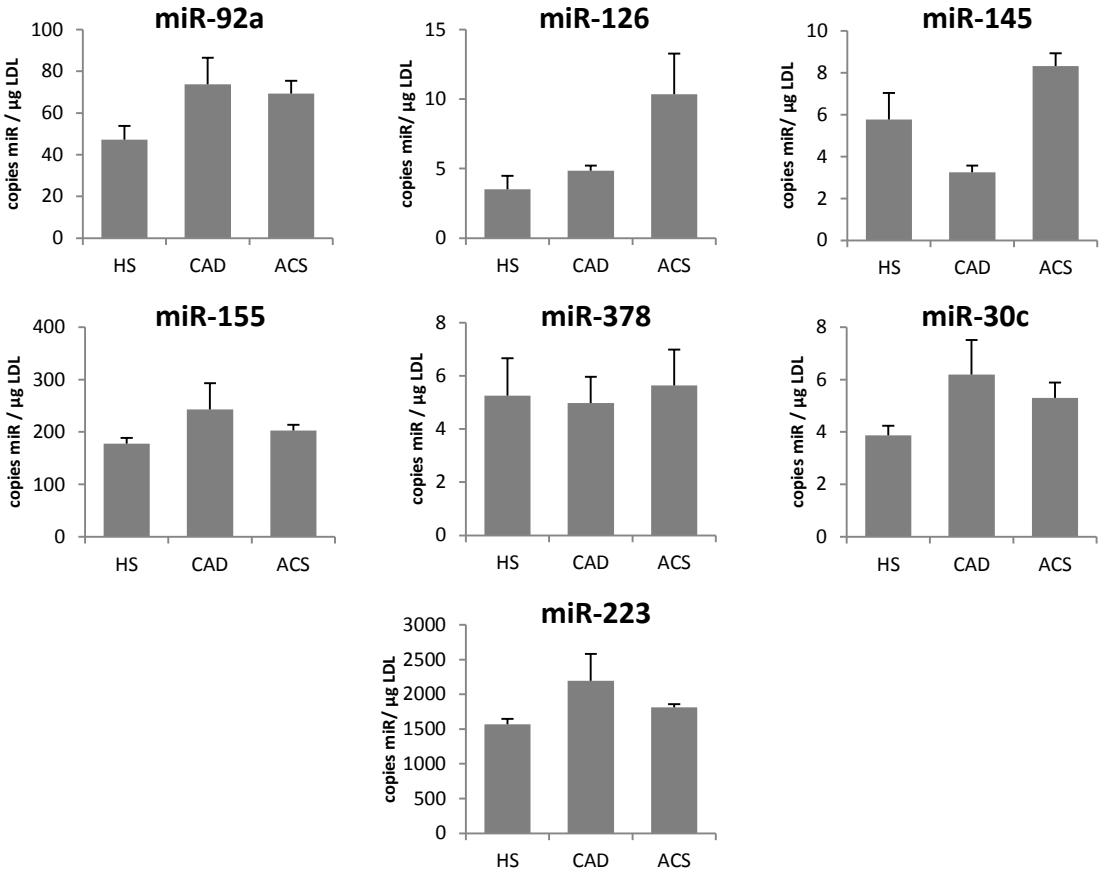
Supplemental Figure 1





Supplemental Figure 1
HDL and Plasma miR levels in patients with cardiovascular disease. Correlation between HDL bound miRs and plasma containing miRs in patients with CAD and ACS (two-sided Spearman correlation). 10µl HDL resulted from 250µl plasma.

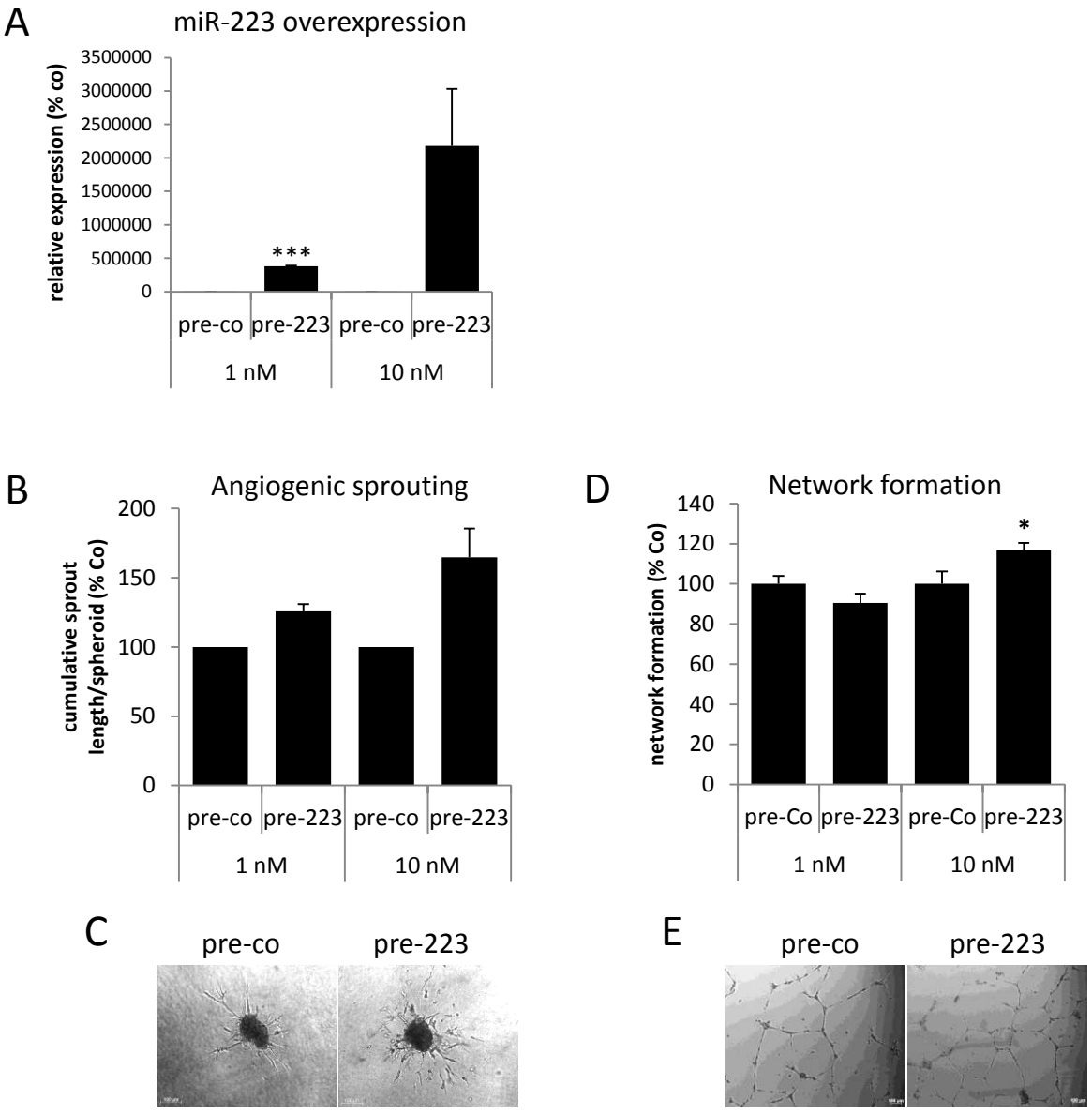
Supplemental Figure 2



Supplemental Figure 2

LDL bound miRs in patients with cardiovascular disease. miR expression determined by qPCR in LDL from control subjects (HS), patients with stable coronary artery disease (CAD) and acute coronary syndrome (ACS), n=5 each. Copy numbers of indicated miRs per µg LDL calculated from a standard curve for each miR with known concentrations.

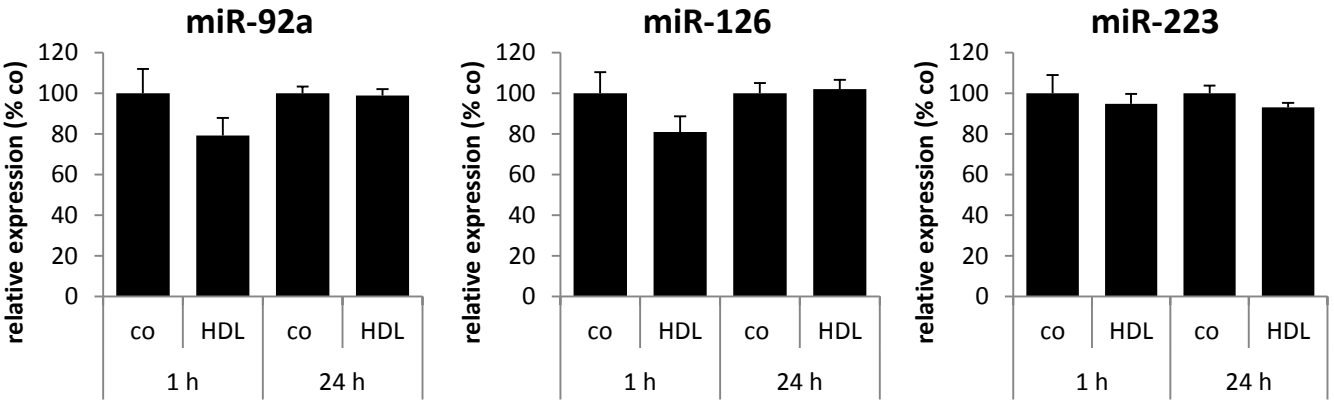
Supplemental Figure 3



Supplemental Figure 3

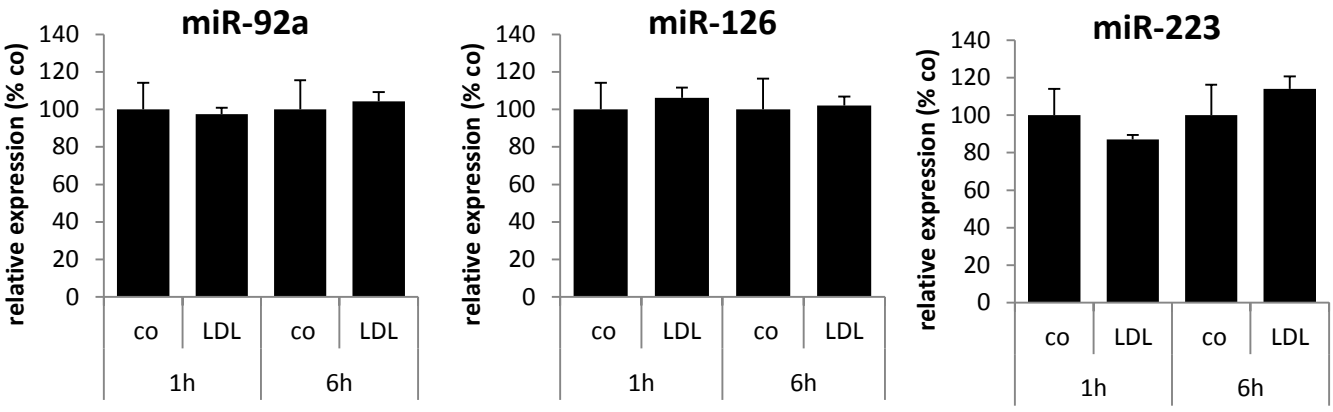
Effect of miR-223 overexpression on angiogenesis in endothelial cells. A, HUVEC were transfected with miR-223 precursor (pre-223) or precursor control (pre-co) in indicated concentrations. Data were normalized to RNU6. n=4. **B**, Sprout formation in Spheroid assay (n=10-12 spheroids/experiment, n=3 experiments. **C**, Representative images of Spheroids (10mM precursors). **D**, Vascular network formation of HUVEC matrigel (n=6 experiments). *p<0.05 versus co (t-test). **E**, Representative images of vascular networks (10mM precursors).

Supplemental Figure 4



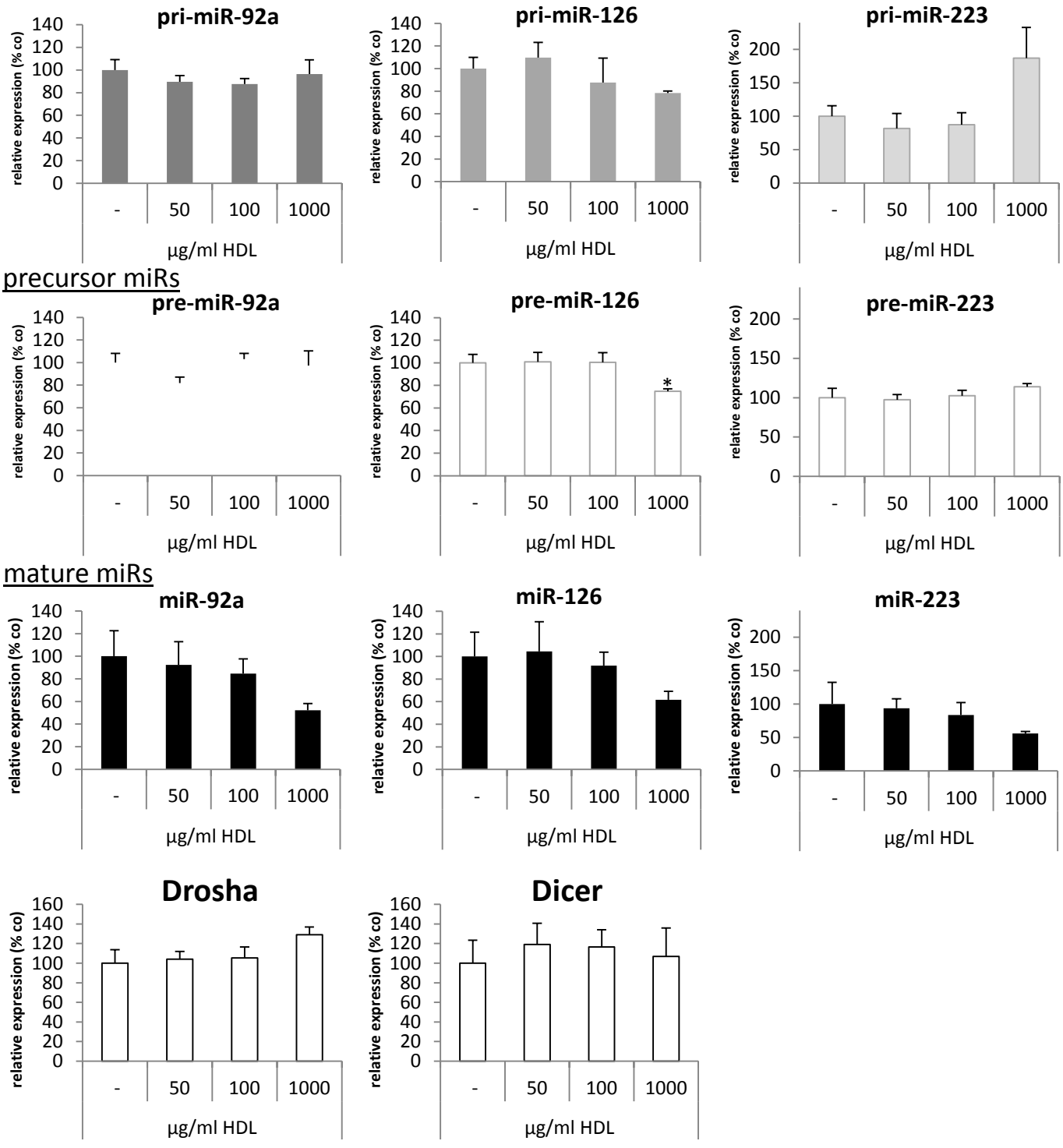
Supplemental Figure 4
HDL bound miR delivery to endothelial cells.
Relative expression of miR-92a, miR-126 and miR-223 after addition of 1 mg/ml HDL (HDL) or no addition of HDL (co) for 1 or 24 hours, (n=5-10). Data were normalized to RNU6.

Supplemental Figure 5



Supplemental Figure 5
LDL bound miR delivery to endothelial cells.
Relative expression of miR-92a, miR-126 and miR-223 after addition of 50 µg/ml LDL (LDL) or no addition of LDL (co) for 1 or 24 hours, (n=5-10). Data were normalized to RNU6.

Supplemental Figure 6



Supplemental Figure 6
Influence of HDL on miR biogenesis in endothelial cells
Relative expression of primary (pri-), precursor (pre-), mature miRs and Drosha and Dicer mRNAs after addition of 50/100/1000 µg/ml HDL or no addition of HDL (-) for 1 hour. Data were normalized to ribosomal P0 or RNU6. *p<0.05 (student´s ttest)

Supplemental References

1. Korff T, Augustin HG. Integration of endothelial cells in multicellular spheroids prevents apoptosis and induces differentiation. *J Cell Biol*. 1998;143:1341-1352
2. Diehl F, Rossig L, Zeiher AM, Dimmeler S, Urbich C. The histone methyltransferase mll is an upstream regulator of endothelial-cell sprout formation. *Blood*. 2007;109:1472-1478